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(54) Title: OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE

(57) Abstract

The present invention concerns long half-life derivative of the obesity protein OB. The invention specifically concerns OB protein-immunoglobulin chimeras and polyethylene glycol (PEG)-OB derivatives, which have extended half-life as compared to the corresponding native OB proteins. The invention further relates to methods for appetite and/or weight reduction and for treating other physiological conditions by using the long half-life derivatives of OB.

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OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE

Field of the Invention

The invention concerns long half-life derivatives of the OB protein. In particular, the invention concerns OB protein-immunoglobulin chimeras, and other long half-life derivatives of the OB protein, and compositions comprising and methods for administering them. The invention further relates to a method for treating obesity by administering a long half-life variant of the OB protein, such as, an OB protein-immunoglobulin chimera.

Background of the Invention

Obesity is the most common nutritional disorder which, according to recent epidemiologic studies, affects about one third of all Americans 20 years of age or older. Kuczmarski *et al.*, J. Am. Med. Assoc. 272, 205-11 (1994). Obesity is responsible for a variety of serious health problems, including cardiovascular disorders, type II diabetes, insulin-resistance, hypertension, hypertriglyceridemia, dyslipoproteinemia and some forms of cancer. Pi-Sunyer, F.X., Anns. Int. Med. 119, 655-60 (1993); Colfitz, G.A., Am. J. Clin. Nutr. 55, 503S-507S (1992). A single-gene mutation (the obesity or "*ob*" mutation) has been shown to result in obesity and type II diabetes in mice. Friedman, Genomics 11, 1054-1062 (1991). Zhang *et al.*, Nature 372, 425-431 (1994) have recently reported the cloning and sequencing of the mouse *ob* gene and its human homologue, and suggested that the *ob* gene product may function as part of a signalling pathway from adipose tissue that acts to regulate the size of the body fat depot. Parabiosis experiments performed more than 20 years ago predicted that the genetically obese mouse containing two mutant copies of the *ob* gene (*ob/ob* mouse) does not produce a satiety factor which regulates its food intake, while the diabetic (*db/db*) mouse produces but does not respond to a satiety factor. Coleman and Hummal, Am. J. Physiol. 217, 1298-1304 (1969); Coleman, Diabetol 9, 294-98 (1973). Recent reports by three independent research teams have demonstrated that daily injections of recombinant OB protein inhibit food intake and reduce body weight and fat in grossly obese *ob/ob* mice but not in *db/db* mice (Pelleymounter *et al.*, Science 269, 540-43 [1995]; Halaas *et al.*, Science 269, 543-46 [1995]; Campfield *et al.*, Science 269, 546-49 [1995]), suggesting that the *ob* protein is such a satiety factor as proposed in early cross-circulation studies. The results of these first studies leave many questions unanswered, and show a number of as yet unresolved discrepancies. For example, while modest effects of daily injections of the *ob* protein on food intake and body weight were reported in lean mice, there was a significant reduction in body fat as assessed by carcass composition in one (Halaas *et al.*, *supra*) but not in another (Pelleymounter *et al.*, *supra*) of these reports, despite equivalent decreases in body weight. Furthermore, Pelleymounter *et al.*, *supra* observed that, for reasons unknown, *ob/ob* mice treated with a 0.1 mg/kg/day dose of the OB protein actually increased their body weight by 17.13 %, while the weight reduction in the obese mice that received a 1 mg/kg/day dose of *ob* was rather moderate. The receptor or receptors of the *ob* protein are as of yet unidentified. While the existence of peripheral receptors cannot be ruled out at this time, the recent report that an increased

expression of the *ob* gene in adipose tissue of mice with hypothalamic lesions does not result in a lean phenotype suggests that the OB protein does not act directly on fat cells. Maffei *et al.*, Proc. Natl. Acad. Sci. 92, 6957-60 (1995). Researchers suggest that at least one OB receptor is localized in the brain. The identification and expression cloning of a leptin receptor (OB-R) was reported by Tartaglia *et al.*, Cell 83, 1263-71 (1995). Various isoforms of a leptin receptor are described by Cioffi *et al.*, Nature 2, 585-89 (1996). A human hematopoetin receptor, which might be a receptor of the OB protein, is described in PCT application Publication No. WO 96/08510, published 21 March 1996. A receptor of the OB protein is disclosed in Tartaglia *et al.*, Cell 83, 1263-71 (1995).

Summary of the Invention

The present invention is based on the observation that the OB protein is significantly more effective at reducing body weight and adipose tissue weight when delivered as a continuous subcutaneous infusion than when the same dose is delivered as a daily subcutaneous injection. The invention is further based on the unexpected finding that a chimeric protein, in which the OB polypeptide is fused to an immunoglobulin constant domain, is strikingly more potent in reducing the body weight and adipose depots than native human OB, when both proteins are administered by subcutaneous injection once a day. The latter observation is particularly surprising since the OB protein-immunoglobulin chimera due to its large molecular weight, is not expected to be able to cross the blood-brain barrier, and reach the OB receptor which has been believed to be located in the brain.

In one aspect, the invention concerns long half-life derivatives of an OB protein capable of reducing body weight and/or food intake in an individual treated. The invention further concerns compositions containing such derivatives, and their administration for reducing body weight and/or food intake.

In another aspect, the invention concerns chimeric polypeptides comprising an OB protein amino acid sequence capable of binding to a native OB receptor linked to an immunoglobulin sequence (briefly referred to as OB-immunoglobulinchimeras or immunoadhesins). In a specific embodiment, the chimeric polypeptides comprise a fusion of an OB amino acid sequence capable of binding a native OB receptor, to an immunoglobulin constant domain sequence. The OB portion of the chimeras of the present invention preferably has sufficient amino acid sequences from a native OB protein to retain the ability to bind to and signal through a native OB receptor. Most preferably, the OB protein retains the ability to reduce body weight when administered to obese human or non-human subjects. The OB polypeptide is preferably human, and the fusion is preferably with an immunoglobulin heavy chain constant domain sequence. In a particular embodiment, the association of two OB polypeptide-immunoglobulin heavy chain fusions (e.g., via covalent linkage by disulfide bond(s)) results in a homodimeric immunoglobulin-like structure. An immunoglobulin light chain may further be associated with one or both of the OB-immunoglobulin chimeras in the disulfide-bonded dimer to yield a homotrimeric or homotetrameric structure.

The invention further concerns nucleic acid encoding chimeric polypeptide chains of the present invention, expression vectors containing DNA encoding such molecules, transformed host cells, and methods for the production of the molecules by cultivating transformant host cells.

Although the long half-life derivatives of the present invention are particularly useful for reducing body weight and/or food intake, they can generally be used for the treatment of conditions associated with the abnormal expression or function of the OB gene and/or to elicit biological responses mediated by an OB receptor. Thus, the OB derivatives of the present invention may be used to treat bulemia, to reduce insulin levels, e.g. in Type I or II diabetic patients, and as mitogens of various cell types expressing an OB receptor. All these and related uses are within the scope of the present invention.

In another embodiment, the invention concerns the purification of an OB receptor by using an OB protein-immunoglobulin chimera.

Brief Description of the Figures

Figure 1 top -- Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The data shown are the mean body weight of each group, in grams, n = 4 mice/point.

Figure 1 bottom -- The mean weight of the retroperitoneal fat pads are shown. Continuous subcutaneous infusions of the OB protein were also more effective than daily subcutaneous injections at reducing adipose tissue weight.

Figure 2 top -- Obese female *ob/ob* mice were treated with human OB protein (hOB) or with a human OB-IgG-1 fusion protein (hOB-IgG-1). The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, n = 3 mice/bar except for the hOB 0.19 mg/kg/day by injection group, where n = 4, and PBS injection group, where n = 1.

Figure 2 bottom -- The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, n = 1/bar.

Figure 3 top and bottom -- Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 fusion protein by daily subcutaneous injections for 7 days. The data are depicted as in Figure 2, with n = 4 for all treatment groups.

Figure 4 top - - Obese female *ob/ob* mice were treated with human protein (hOB) or with PEG-hOB. The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, n = 3-4 mice/bar except for the PBS injection group, where n = 1. The materials were injected daily subcutaneously. The "PEG 1X" and "PEG 2X" refer to the ratio of the PEG reagent to protein in the preparation of the molecule.

Figure 4 bottom - - The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, n = 3-4/bar.

Figure 5 - - Obese (*ob/ob*) female mice were treated with either the hOB-IgG fusion protein, native hOB, or hCD4-IgG by daily subcutaneous injections for 7 days. n = 6 for all treatment groups, except hOB at 3.8 mg/kg/d, where n = 2. Again it was observed that the fusion protein was more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

Figure 6 - - The nucleotide sequence (SEQ. ID. NO:1) and the amino acid sequence (SEQ. ID. NO: 2) of the human OB-IgG-1 chimera of Example 1.

Detailed Description of the Invention

A. Definitions

The term "obesity" is used to designate a condition of being overweight associated with excessive bodily fat. The desirable weight for a certain individual depends on a number of factors including sex, height, age, overall built, etc. The same factors will determine when an individual is considered obese. The determination of an optimum body weight for a given individual is well within the skill of an ordinary physician.

The phrase "long half-life" and grammatical variants thereof, as used in connection with OB derivatives, concerns OB derivatives having a longer plasma half-life and/or slower clearance than a corresponding native OB protein. The long half-life derivatives preferably will have a half-life at least about 1.5-times longer than a native OB protein; more preferably at least about 2-times longer than a native OB protein, more preferably at least about 3-time longer than a native OB protein. The native OB protein preferably is that of the individual to be treated.

The terms "OB", "OB polypeptide" , "OB protein" and their grammatical variants are used interchangeably and refer to "native" or "native sequence" OB proteins (also known as "leptins") and their functional derivatives. The OB polypeptides have the typical structural features of cytokines, i.e. polypeptides released by one cell population which act on another cell as intercellular mediators, such as, for example, growth hormones, insulin-like growth factors, interleukins, insulin, glycoprotein hormones such as, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), tumor necrosis factor-α and -β (TNF-α and -β), nerve growth factors, such as NGF-β, PDGF, transforming growth factors (TGFs) such as, TGF-α and TGF-β, insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), erythropoietin, osteoinductive factors, interferons (IFNs) such as, IFN-α, IFN-β and IFN-γ, colony stimulating factors (CSFs) such as, M-CSF, GM-CSF, and G-CSF, interleukins (ILs) such as, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and other polypeptide factors.

The terms "native" and "native sequence" OB polypeptide are used to refer to an OB polypeptide from any animal species (e.g. human, murine, rabbit, cat, cow, sheep, chicken, porcine, equine, etc.), as occurring in nature, including naturally-occurring alleles, deletion, substitution and/or insertion variants, as currently known or as might be identified in the future, provided that they retain the ability to bind to and, preferably, signal through the OB receptor. Thus, a native human OB polypeptide includes the amino acid sequence between the N-terminus and the cysteine (Cys) at position 167 of the amino acid sequence shown in Figure 6 (see also SEQ. ID. NO: 2 and Figure 6 of Zhang *et al.*, *supra*), and naturally occurring variants of this protein, as currently known or might be identified in the future. Similarly, a "native" or "native sequence" murine OB polypeptide has the amino acid sequence shown in Figure 6 of Zhang *et al.*, *supra*, and naturally occurring variants of that polypeptide, as currently known or might be identified in the future. The definition specifically includes variants with or without a glutamine at amino acid position 49, using the amino acid numbering of Zhang *et al.*, *supra*. The terms "native" and "native sequence" OB polypeptide include the native proteins with or without the initiating N-terminal methionine (Met), and with or without the native signal sequence, either in monomeric or in dimeric form. The native human and murine OB polypeptides known in the art are 167 amino acids long, contain two conserved cysteines, and have the features of a secreted protein. The polypeptide is largely

hydrophilic, and the predicted signal sequence cleavage site is at position 21, using the amino acid numbering of Zhang *et al.*, *supra*. The overall sequence homology of the human and murine sequences is about 84%. The two proteins show a more extensive identity in the N-terminal region of the mature protein, with only four conservative and three non-conservative substitutions among the residues between the signal sequence cleavage site and the conserved Cys at position 117. The molecular weight of OB proteins is about 16 kD in a monomeric form.

A "functional derivative" of a native polypeptide is a compound having a qualitative biological property in common with the native polypeptide. A functional derivative of an OB polypeptide is a compound that has a qualitative biological property in common with a native (human or non-human) OB polypeptide. "Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including humans), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a corresponding native polypeptide.

"Fragments" comprise regions within the sequence of a mature native OB polypeptide. Preferred fragments of OB polypeptides include the C-terminus of the mature protein, and may contain relatively short deletion(s) at the N-terminus and in other parts of the molecule not required for receptor binding and/or for structural integrity.

The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a native polypeptide, whereas the term "variant" refers to amino acid sequence variants within this definition.

"Biological property" in the context of the definition of "functional derivatives" is defined as either 1) immunological cross-reactivity with at least one epitope of a native polypeptide (e.g. a native OB polypeptide of any species), or 2) the possession of at least one adhesive, regulatory or effector function qualitatively in common with a native polypeptide.

Preferably, the functional derivatives are polypeptides which have at least about 65% amino acid sequence identity, more preferably about 75% amino acid sequence identity, even more preferably at least about 85% amino acid sequence identity, most preferably at least about 95% amino acid sequence identity with a native polypeptide. In the context of the present invention, functional derivatives of native sequence human OB polypeptides preferably show at least 95% amino acid sequence identity with the native OB proteins, and are not immunogenic in the human.

Amino acid sequence identity or homology is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology.

Immunologically cross-reactive as used herein means that the candidate (poly)peptide is capable of competitively inhibiting the qualitative biological activity of a corresponding native polypeptide having this activity with polyclonal antibodies or antisera raised against the known active molecule. Such antibodies and antisera are prepared in conventional fashion by injecting an animal such as a goat or rabbit, for example,

subcutaneously with the known native OB protein in complete Freud's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freud's.

The term "isolated OB polypeptide" and grammatical variants thereof refer to OB polypeptides (as hereinabove defined) separated from contaminant polypeptides present in the human, other animal species, or in other source from which the polypeptide is isolated.

In general, the term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a reference (e.g. native sequence) polypeptide. The amino acid alterations may be substitutions, insertions, deletions or any desired combinations of such changes in a native amino acid sequence.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native amino acid sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

"Covalent derivatives" include modifications of a native polypeptide or a fragment thereof with an organic proteinaceous or non-proteinaceous derivatizing agent, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sites or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the OB-immunoglobulin chimeras of the present invention. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosine or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

The terms "DNA sequence encoding", "DNA encoding" and "nucleic acid encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the

foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancer.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

Native immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one and (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, J. Mol. Biol. 186, 651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82, 4592-4596 [1985]).

Depending on the amino acid sequence of the constant region of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and

IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. IgA-1 and IgA-2 are monomeric subclasses of IgA, which usually is in the form of dimers or larger polymers. Immunocytes in the gut produce mainly polymeric IgA (also referred to poly-IgA including dimers and higher polymers). Such poly-IgA contains a disulfide-linked polypeptide called the "joining" or "J" chain, and can be transported through the glandular epithelium together with the J-containing polymeric IgM (poly-IgM), comprising five subunits.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

B. OB protein-immunoglobulin chimeras (immunoadhesins)

Immunoadhesins are chimeric antibody-like molecules that combine the functional domain(s) of a binding protein (usually a receptor, a cell-adhesion molecule or a ligand) with the an immunoglobulin sequence. The most common example of this type of fusion protein combines the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. This type of molecule is called an "immunoadhesin", because it combines "immune" and "adhesion" functions; other frequently used names are "Ig-chimera", "Ig-" or "Fc-fusion protein", or "receptor-globulin."

To date, more than fifty immunoadhesins have been reported in the art. Immunoadhesins reported in the literature include, for example, fusions of the T cell receptor (Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA 84, 2936-2940 [1987]); CD4 (Capon *et al.*, Nature 337, 525-531 [1989]; Traunecker *et al.*, Nature 339, 68-70 [1989]; Zettmeissl *et al.*, DNA Cell Biol. USA 9, 347-353 [1990]; Byrn *et al.*, Nature 344, 667-670 [1990]); L-selectin (homing receptor) (Watson *et al.*, J. Cell. Biol. 110, 2221-2229 [1990]; Watson *et al.*, Nature 349, 164-167 [1991]); E-selectin [Mulligan *et al.*, J. Immunol. 151, 6410-17 [1993]; Jacob *et al.*, Biochemistry 34, 1210-1217 [1995]); P-selectin (Mulligan *et al.*, *supra*; Hollenbaugh *et al.*, Biochemistry 34, 5678-84 [1995]); ICAM-1 (Stauton *et al.*, J. Exp. Med. 176, 1471-1476 [1992]; Martin *et al.*, J. Virol. 67, 3561-68 [1993]; Roep *et al.*, Lancet 343, 1590-93 [1994]); ICAM-2 (Damle *et al.*, J. Immunol. 148, 665-71 [1992]); ICAM-3 (Holness *et al.*, J. Biol. Chem. 270, 877-84 [1995]); LFA-3 (Kanner *et al.*, J. Immunol. 148, 2-23-29 [1992]); L1 glycoprotein (Doherty *et al.*, Neuron 14, 57-66 [1995]); TNF-R1 (Ashkenazi *et al.*, Proc. Natl. Acad. Sci. USA 88, 10535-539 [1991]; Lesslauer *et al.*, Eur. J. Immunol. 21, 2883-86 [1991]; Peppel *et al.*, J. Exp. Med. 174, 1483-1489 [1991]); TNF-R2 (Zack *et al.*, Proc. Natl. Acad. Sci. USA 90, 2335-39 [1993]; Wooley *et al.*, J.

Immunol. 151, 6602-07 [1993]); CD44 [Aruffo *et al.*, Cell 61, 1303-1313 (1990)]; CD28 and B7 [Linsley *et al.*, J. Exp. Med. 173, 721-730 (1991)]; CTLA-4 [Lisley *et al.*, J. Exp. Med. 174, 561-569 (1991)]; CD22 [Stamenkovic *et al.*, Cell 66. 1133-1144 (1991)]; NP receptors [Bennett *et al.*, J. Biol. Chem. 266, 23060-23067 (1991)]; IgE receptor α [Ridgway and Gorman, J. Cell. Biol. 115, abstr. 1448 (1991)]; HGF receptor [Mark, M.R. *et al.*, 1992, J. Biol. Chem. submitted]; IFN-γR α- and β-chain [Marsters *et al.*, Proc. Natl. Acad. Sci. USA 92, 5401-05 [1995]); trk-A, -B, and -C (Shelton *et al.*, J. Neurosci. 15, 477-91 [1995]); IL-2 (Landolfi. J. Immunol. 146, 915-19 [1991]); IL-10 (Zheng *et al.*, J. Immunol. 154, 5590-5600 [1995]).

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the 'adhesin' protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the OB-immunoglobulin chimeras of the present invention, nucleic acid encoding the desired OB polypeptide will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible. Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the OB-immunoglobulin chimeras.

In a preferred embodiment, the sequence of a native, mature OB polypeptide, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. IgG-1. It is possible to fuse the entire heavy chain constant region to the OB sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet *et al.*, *supra*], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the OB polypeptide sequence is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, or IgG-3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the OB-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or -tetramers (WO 91/08298). Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Various exemplary assembled OB-immunoglobulin chimeras within the scope herein are schematically diagrammed below:

(a) AC_L-AC_L;

(b) AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H];

(c) AC_L-AC_H-[AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(d) AC_L-V_HC_H-[AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H];

(e) V_LC_L-AC_H-[AC_L-V_HC_H, or V_LC_L-AC_H]; and

(f) $[A\text{-}Y]_n$-$[V_LC_L\text{-}V_HC_H]_2$,

wherein

each A represents identical or different OB polypeptide amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed as being present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the OB amino acid sequences can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the OB polypeptide sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom, H. R. *et al.*, Mol. Immunol. 28, 1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an OB protein-immunoglobulin heavy chain fusion polypeptide, or directly fused to the OB polypeptide. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the OB-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Method suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG-1 and IgG-3 immunoglobulin sequences is preferred. A major advantage of using IgG-1 is that IgG-1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG-3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG-3 hinge is longer and more flexible, so it can accommodate larger 'adhesin' domains that may not fold or function properly when fused to IgG-1. Possible IgG-based immunoadhesin structures are shown in Fig. 3a-c. While IgG immunoadhesins are typically

mono- or bivalent, other Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. A typical IgM-based multimeric immunoadhesin is illustrated in Figure 3d. Multimeric immunoadhesins are advantageous in that they can bind their respective targets with greater avidity than their IgG-based counterparts. Reported examples of such structures are CD4-IgM (Traunecker *et al.*, *supra*); ICAM-IgM (Martin *et al.*, J. Virol. 67, 3561-68 [1993]); and CD2-IgM (Arulanandam *et al.*, J. Exp. Med. 177, 1439-50 [1993]).

For OB-Ig immunoadhesins, which are designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG-1, IgG-2 and IgG-4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG-4 does not activate complement, and IgG-2 is significantly weaker at complement activation than IgG-1. Moreover, unlike IgG-1, IgG-2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG-3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG-1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG-3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a γ3 immunoadhesin is greater than that of a γ1 immunoadhesin.

In designing the OB-Ig immunoadhesins of the present invention regions that are not required for receptor binding, the structural integrity (e.g. proper folding) and/or biological activity of the molecule, may be deleted. In such structures, it is important to place the fusion junction at residues that are located between domains, to avoid misfolding. With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the "adhesin" (OB) part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP of the IgG1 hinge region.

OB-Ig immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the OB portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA 84, 2936-2940 [1987]; Aruffo *et al.*, Cell 61, 1303-1313 [1990]; Stamenkovic *et al.*, Cell 66, 1133-1144 [1991]). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. Murine OB cDNA can, for example, be obtained by PCR from a mouse adipose tissue cDNA library (Clontech), using primers designed based on the sequence of Zhang *et al.* Human OB cDNA can be obtained in a similar manner. Alternatively, the mouse OB gene can be used as a probe to isolate human adipose tissue cDNA clones (Clontech), e.g. from a λgt11 library, as described by Zhang *et al.* The cDNAs encoding the 'adhesin' and the Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For

expression in mammalian cells pRK5-based vectors (Schall *et al.*, Cell 61, 361-370 [1990]), pRK7-vectors and CDM8-based vectors (Seed, Nature 329, 840 [1989]) are preferred. (pRK7 is identical to pRK5 except that the order of the endonuclease restriction sites in the polylinker region between ClaI and HindIII is reversed. See U.S. Patent No. 5,108,901 issued 28 April 1992.). The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller and Smith, Nucleic Acids Res. 10, 6487 [1982]; Capon *et al.*, Nature 337, 525-531 [1989]). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR technique can be used to join the two parts of the molecule in-frame with an appropriate vector.

Immunoadhesins can be expressed efficiently in a variety of host cells, including myeloma cell lines, Chinese Hamster ovary (CHO) cells, monkey COS cells, human embryonic kidney 293 cells, and baculovirus infected insect cells. In these systems, the immunoadhesin polypeptides are assembled and secreted into the cell culture medium. Yeasts, e.g. Saccharomyces cerevisiae, Pichia pastoris, etc., and bacterial cells, preferably E. coli, can also be used as hosts. The OB-immunoglobulin chimeras can be expressed in yeast, for example, similarly to the process described for the expression of the OB proteins by Leiber *et al.*, Crit. Res. Food Sci. Nutr. 33, 351 (1993); Friedman and Leibel, Cell 69, 217 (1992); and Beavis and Chait, Proc. Natl. Acad. Sci. USA 87, 6873 (1990). Thus, the coding sequences can be subcloned into a yeast plasmid, such as the yeast expression plasmid pPIC.9 (Invitrogen). This vector directs secretion of heterologous proteins from the yeast into the culture media. According to Halaas *et al.*, *supra*, expression of mouse and human OB genes in Saccharomyces cerevisiae transformed with this vector yields a secreted 16-kD protein, which is an unprocessed OB protein lacking the signal sequence. Expression of the mouse or human OB-immunoglobulin chimeras in E. coli can, for example, be performed on the analogy of the procedure described by Halaas *et al.*, *supra*. The coding sequences of mouse and human OB-immunoglobulin chimeras can be subcloned into the PET15b expression vector (Novagen) and expressed in E. coli (BL21 (DE3)pIYsS) through use of the T7 E. coli RNA polymerase system. Alternatively, the fusion protein can be expressed in E. coli by inserting the coding sequence in frame with the secretion sequence of the E. coli heat stable enterotoxin II, downstream of the E. coli alkaline phosphatase promoter (Chang *et al.*, Gene 55, 189-96 [1987]).

The choice of host cell line for the expression of OB-Ig immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5- and pRK7-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. This method is illustrated in the examples. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, Cell 61, 1303-1313 (1990); Zettmeissl *et al.*, DNA Cell Biol. (US) 9, 347-353 (1990)]. If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5- or pRK7-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels

of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts [Gascoigne *et al.*, 1987, *supra*; Martin *et al.*, J. Virol. 67, 3561-3568 (1993)].

The expression of immunoadhesins with more complex oligomeric structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne *et al.*, *supra*; Martin *et al.*, J. Immunol. 67, 3561-68 [1993]).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human γ1, γ2, or γ4 heavy chains [Lindmark *et al.*, J. Immunol. Meth. 62, 1-13 (1983)]. Protein G is recommended for all mouse isotypes and for human γ3 [Guss *et al.*, EMBO J. 5, 15671575 (1986)]. The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human γ1 molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography [Hutchens and Porath, Anal. Biochem. 159, 217-226 (1986)] and immobilized metal chelate chromatography [Al-Mashikhi and Makai, J. Dairy Sci. 71, 1756-1763 (1988)]. In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature. Microheterogeneity of charge can also be a factor for immunoadhesins in which the adhesin portion of the molecule is glycosylated and contains sialic acid. A specific purification protocol is described in the examples.

Results with the numerous immunoadhesins produced so far show that the fusion of the adhesin portion to an Fc region usually does not perturb the folding of the individual domains. Both the adhesin and the immunoglobulin regions appear to fold correctly, and the Fc portion retins many of the effector functions that are characteristic of antibodies, such as binding to Fc receptors.

Methods generally applicable for the construction, expression and purification of immunoadhesins are described, for example, in U.S. Patent Nos. 5,225,538 (issued 6 July 1993) and 5,455,165 (issued 30 October 1995), the disclosures of which are hereby expressly incorporated by reference. Immunoadhesin construction,

expression, purification and various immunoadhesins designs are also described in the review articles by Ashkenazi and Chamow, Methods in Enzymology 8, 104-115 (1995), and Peach and Linsley, Methods in Enzymology 8, 116-123 (1995), the disclosures of which, along with the references cited therein, is hereby expressly incorporated by reference.

C. **Other long half-life OB derivatives**

Other derivatives of the OB proteins, which possess a longer half-life than the native molecules comprise the OB protein or an OB-immunoglobulinchimera, covalently bonded to a nonproteinaceouspolymer. The nonproteinaceouspolymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyelkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrins, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general, the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the OB protein or to the OB-immunoglobulin chimeras though a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar residues of the OB protein or OB-immunoglobulinchimera to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or via versa.

The covalent crosslinking site on the OB protein or OB-Ig includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde;PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylcloroformate or P-nitrophenylcloroformate activated PEG.) Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, e.g. metaperiodate, or enzymes, e.g. glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.*, P.N.A.S., 71, 3537-41 (1974) or Bayer *et al.*, Methods in Enzymology 62, 310 (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, e.g. by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

"Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein (e.g. an OB-immunoglobulin chimera), the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, e.g. PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, Anal Biochem. 131, 25-33 [1983]) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, J. Polym. Sci. Polym. Chem. Ed. 22, 341-52 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

Functionalized PEG polymers to modify the OB protein or OB-Ig chimeras of the present invention are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (lysine or cysteine), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkeage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (e.g. containing

one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids.

D. The use of the OB-immunoglobulin chimeras and other long half-life derivatives

The OB-immunoglobulin chimeras and other long half-life OB derivatives of the present invention are useful for weight reduction, and specifically, in the treatment of obesity and other disorders associated with the abnormal expression or function of the OB gene. Our studies indicate that the OB-immunoglobulin chimeras and other long half-life OB derivatives, e.g. PEGylated OB, reduce the food intake and increase the energy use of animals treated, and are therefore very effective in reducing the weight of both obese and normal subjects. For testing purposes, the molecules of the present invention may be dissolved in phosphate-buffered saline (PBS) (pH 7.4), and administered by intravenous or subcutaneous injection, or infusion.

The long acting OB-derivatives of the present invention may further be used to treat other metabolic disorders such as diabetes and bulimia. The OB protein has been shown to reduce insulin levels in animals, and could be useful to reduce excessive levels of insulin in human patients. The reduction of insulin levels in obese or non-obese patients (e.g. Type I or II diabetics) could restore or improve the insulin-sensitivity of such patients.

In addition, the long half-life OB-derivatives can be used for the treatment of kidney ailments, hypertension, and lung disfunctions, such as emphysema. The OB protein might also cause a mitogenic response in receptor-bearing tissues, acting as a growth factor for these cells.

Therapeutic formulations of the present invention are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, etc. routes. Sustained released formulations are also foreseen. Suitable examples of sustained release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Patent 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman et al., 1983, "Biopolymers" 22 (1): 547-556), poly (2-hydroxyethyl-methacrylate) (R. Langer, et al., 1981, "J. Biomed. Mater. Res." 15: 167-277 and R. Langer, 1982, Chem. Tech." 12: 98-105), ethylene vinyl acetate (R. Langer et al., Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988A). Sustained release compositions also include liposomes. Liposomes containing a molecule within the scope of the present invention are prepared by methods known per se: DE 3,218,121A; Epstein et al., 1985, "Proc. Natl. Acad. Sci. USA" 82: 3688-3692; Hwang et al., 1980, "Proc. Natl. Acad. Sci. USA" 77: 4030-4034; EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142641A; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324A. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal therapy.

An effective amount of a molecule of the present invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assay techniques. If the purpose of the treatment is weight reduction, the therapy is normally continued until a desired body weight is reached.

Non-therapeutic uses of the OB protein-immunoglobulin fusions of the present invention include their use to identify and purify OB receptors. The identification and expression cloning of an OB receptor, using an OB protein-immunoadhesin is described in a Reference Example hereinbelow.

The invention will be further illustrated by the following non-limiting examples.

Example 1

Expression of OB- immunoadhesins

Using protein engineering techniques, the human OB protein was expressed as a fusion with the hinge, CH2 and CH3 domains of IgG-1. DNA constructs encoding the chimera of the human OB protein and IgG-1 Fc domains were made with the Fc region clones of human IgG-1. Human OB cDNA was obtained by PCR from human fat cell dscDNA (Clontech Buick-Clone cDNA product). The source of the IgG-1 cDNA was the plasmid pBSSK-CH_2CH_3. The chimera contained the coding sequence of the full length OB protein (amino acids 1-167 in Figure 5) and human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region (Kabat *et al.*, Sequences of Proteins of Immunological

Interest 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. There was an insert of codons for three amino acids (GlyValThr) between the OB and IgG-1 coding sequences. If necessary, this short linker sequence can easily be deleted, for example by site directed deletion mutagenesis, to create an exact junction between the coding sequences of the OB protein and the IgG-1 hinge region. The coding sequence of the OB-IgG-1 immunoadhesin was subcloned into the pRK5-based vector pRK5tk-neo which contains a neomycine selectable marker, for transient expression in 293 cells using the calcium phosphate technique (Suva *et al.*, Science 237, 893-896 [1987]). 293 cells were cultured in HAM's : Low Glucose DMEM medium (50:50), containing 10% FBS and 2 mM L-Gln. For purification of OB-IgG-1 chimeras, cells were changed to serum free production medium PS24 the day after transfection and media collected after three days. The culture media was filtered.

The filtered 293 cell supernatant (400 ml) containing recombinant human OB-IgG-1 was made 1 mM in phenylmethylsulfonylfluoride and 2 µg/ml in aprotinin. This material was loaded at 4 °C onto a 1 x 4.5 cm Protein A agarose column (Pierce catalog # 20365) equilibrated in 100 mM HEPES pH 8. The flow rate was 75 ml/h. Once the sample was loaded, the column was washed with equilibration buffer until the A_{280} reached baseline. The OB-IgG-1 protein was eluted with 3.5 M $MgCl_2$ + 2% glycerol (unbuffered) at a flow rate of 15 ml/h. The eluate was collected with occasional mixing into 10 ml of 100 mM HEPES pH 8 to reduce the $MgCl_2$ concentration by approximately one-half and to raise the pH. The eluted protein was then dialyzed into phosphate buffered saline, concentrated, sterile filtered and stored either at 4°C or frozen at -70 °C. The OB-IgG-1 immunoadhesin prepared by this method is estimated by SDS-PAGE to be greater than 90% pure.

Example 2

Animal studies

A. Materials and Methods

OB protein Production -- Murine OB cDNA was obtained by PCR from an adipocyte cDNA library using primers based on the sequence of Zhang *et al.*, *supra*. Mature OB protein (amino acids 22-167) was expressed in E. coli by inserting the OB coding sequence in frame with the secretion sequence of the E. coli heat-stable enterotoxin II, downstream of the E. coli alkaline phosphatase promoter. Chang *et al.*, Gene 55, 189-96 (1987). After cell lysis, the insoluble fraction was solubilized in 8 M urea buffer pH 8.35 in the presence of 25 mM DTT. Reduced OB protein was purified by size exclusion and reverse phase HPLC, then refolded in the presence of glutathione. Refolded OB protein was purified by reverse phase HPLC and analyzed by SDS-PAGE and amino acid and mass spectrometry analyses.

Preparation of PEG-hOB -- The PEG derivatives of the human PB protein were prepared by reaction of hOB purified by reverse phase chromatography with a succinimidyl derivative of PEG propionic acid (SPA-PEG) having a nominal molecular weight of 10 kD, which had been obtained from Shearwater Polymers, Inc. (Huntsville, AL). After purification of the hOB protein by reverse phase chromatography, an approximately 1-2 mg/ml solution of the protein in 0.1% trifluoroacetic acid and approximately 40% acetonitrile, was diluted with 1/3 to 1/2 volume of 0.2 M borate buffer and the pH adjusted to 8.5 with NaOH. SPA-PEG was added to

the reaction mixture to make 1:1 and 1:2 molar ratios of protein to SPA-PEG and the mixture was allowed to incubate at room temperature for one hour. After reaction and purification by gel electrophoresis or ion exchange chromatography, the samples were extensively dialyzed against phosphate-buffered saline and sterilized by filtration through a 0.22 micron filter. Samples were stored at 4°C. Under these conditions, the PEG-hOB resulting from the 1:1 molar ratio protein to SPA-PEG reaction consisted primarily of molecules with one 10 kD PEG attached with minor amounts of the 2 PEG-containing species. The PEG-hOB from the 1:2 molar reaction consisted of approximately equal amounts of 2 and 3 PEGs attached to hOB, as determined by SDS gel electrophoresis. In both reactions, small amounts of unreacted protein was also detected. This unreacted protein can be efficiently removed by the gel filtration or ion exchange steps as needed. The PEG derivatives of the human OB protein can also be prepared essentially following the aldehyde chemistry described in EP 372,752 published June 13, 1990.

Animal Studies -- All manipulations involving animals were reviewed and approved by Genentech's Institutional Animal Care and Use Committee. Seven to eight week-old genetically obese C57Bl/6J-*ob/ob* (*ob/ob*) female mice were purchase from Jackson Labs (Bar Harbor, ME). Lean female mice of the same genetic background (C57Bl/6) were purchased from Harlan Sprague Dawley (Hollister, CA). Mice were housed in groups 3 - 6 with *ad libitum* access to water and standard mouse chow (Purina 5010; Purina Mills, Richmond, IN) in a temperature-, humidity- and light-controlled (lights on at 06:00h, of at 18:00h) colony room.

Miniosmotic pumps (Alzet model 2002; Alza Corp., Palo Alto, CA) were filled with purified recombinant OB protein (100 µg/kg/day) in sterile phosphate-buffered saline (PBS) or PBS alone under sterile conditions following manufacturer's instructions and incubated overnight in sterile saline at room temperature prior to implantation into mice. Mice were anesthetized with ketamine/xylazine, and miniosmotic pumps were implanted subcutaneously in the midscapular region. Daily subcutaneous injections of purified recombinant OB protein, hOB-IgG-1 fusion protein or PBS were made into the midscapular region of conscious mice. Injections were performed within one hour of lights out. The body weight of each mouse (to the nearest 0.1 gram) and the weight of the food contained in the food bin in each cage (to the nearest 0.1 gram) were recorded within one hour of lights out every one to two days. The data are depicted as the mean ± SEM. The number of animals is as described below and in the Figure legends.

B. Results with continuous subcutaneous infusion of OB protein

Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The results are shown in Figure 1. The upper chart shows that the OB protein is significantly more effective in reducing body weight when delivered as a continuous infusion than when the same dose is delivered in the form of daily subcutaneous injections. The bottom chart shows the same difference in the ability of the OB protein to reduce adipose tissue weight.

C. Results with the OB-IgG-1 chimera

Obese female *ob/ob* mice were treated with human OB protein or with the human OB-IgG-1 chimera. The data are shown in Figure 2. The data presented in the top chart demonstrate that the hOB-IgG-1 fusion

protein is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion. It is noted that the increase in potency would be even more expressed, if the data were converted to molar amounts, as only about one third of the OB-IgG-1 chimera comes from the OB protein. The data further confirm the previous observation that continuous subcutaneous infusion (pump) or the hOB protein is more effective than daily subcutaneous injections (inj) at reducing body weight.

The data shown at the bottom chart of Figure 2 show that the hOB-IgG-1 fusion protein substantially reduced food intake. This result was unexpected as it was assumed that the fusion protein would be too large to cross the blood-brain barrier and exert its effect.

Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 chimera by daily subcutaneous injections for 7 days. The data shown in Figure 3 again demonstrate that the chimera is more effective than the native hOB protein at reducing body weight (top) and food intake (bottom).

In a further experiment, obese (*ob/ob*) female mice were treated with either the hOB-IgG-1 fusion protein, native hOB or hCD4-IgG-1 (control) by daily subcutaneous injections for seven days. The results shown in Figure 5 affirm that the hOB-IgG-1 fusion protein is more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

D. Results with PEG-hOB

Obese female *ob/ob* mice were treated with human OB protein or with PEG derivatives of human OB. The data are shown in Figure 4. The data presented in the top chart demonstrate that PEG-hOB is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion.

The data shown at the bottom chart of Figure 4 show that the PEG-hOB proteins were substantially more effective in reducing food intake than unmodified native hOB.

Reference Example

Identification and cloning of an OB receptor

The OB protein-immunoadhesinof Example 1 was used to detect and expression clone an OB receptor. First, to identify a receptor source, several cell lines were screened with 1 µg/ml OB-IgG-1 fusion by flow cytometry. The detection system which consists of a biotin conjugated secondary antibody followed by streptavidin-phycoerythrinprovides a dramatic amplification of the signal and allows the detection of cells expressing low numbers of receptors. Two cell lines, human embryonic kidney 293 and human lung A549 cells were found to bind OB-IgG-1 but not an Flt-4 control immunoadhesin. Specific binding of OB-IgG-1 to the cells was also demonstrated by the addition of excess of bacterially expressed human OB protein. Addition of 10 µg/ml of human OB completely blocks the binding of OB-IgG-1 to 293 cells.

To isolate a cDNA encoding the OB receptor, COSN cells were transiently transfected with pools of approximately 10^5 clones of an oligo dT primed 293 cell cDNA library in pRK5B. Transfected cells were enriched by panning on plates coated with an anti-human Fc antibody after incubation with OB-IgG-1. After

three rounds of enrichment, 1 of 30 pools gave rise to OB-IgG-1 mediated adherence of COSN cells to the binding plates which could be competed by human leptin. cDNA clones picked randomly from this third round were transfected in pools of 10-20. Individual clones were finally identified after breaking down one pool of 10 that was scoring positive by panning.

Sequence analysis revealed a clone of approximately 5300 bp with an open reading frame encoding a protein of 896 amino acids. The sequence corresponded to a type 1 transmembrane protein with a 22 amino acid long signal peptide, 819 amino acid extracellular domain, 21 amino acid transmembrane domain and a short 34 amino acid intracellular domain. The sequence was found to essentially correspond to the human OB receptor identified and isolated by Tartaglia *et al., supra*, and is identical with a human receptor sequence disclosed in copending application Serial No. 08/585,005 filed January 11, 1996.

While the invention has been illustrated by way of examples, the scope of the invention is not so limited. It will be understood that further modifications and variations are possible without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

All references cited throughout the specification, including the examples, and the references cited therein are hereby expressly incorporated by reference.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: OB Protein Derivatives

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7127 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 150

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC 350

TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA 800

CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

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GACCTCAGCC CTGGGTGCGG GGTCACCGAC AAAACTCACA CATGCCCACC 1450

GTGCCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC CTCTTCCCCC 1500

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CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC 1900

CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC 1950

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CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG 6100

ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT 6150

AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT 6200

ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG 6250

GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT 6300

TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT 6350

GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA 6400

CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT 6450

TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC 6500

TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT 6550

ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA 6600

TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG 6650

CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG 6700

CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG 6750

CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA 6800

GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG 6850

TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT 6900

TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCTGGC 6950

ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA CGCAATTAAT 7000

GTGAGTTACC TCACTCATTA GGCACCCCAG GCTTTACACT TTATGCTTCC 7050

GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT CACACAGGAA 7100

ACAGCTATGA CCATGATTAC GAATTAA 7127

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 397 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr
1 5 10 15

Leu Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp
20 25 30

Thr Lys Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile
35 40 45

Ser His Thr Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu
50 55 60

Asp Phe Ile Pro Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met
65 70 75

Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile Leu Thr Ser Met Pro
80 85 90

Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu Glu Asn Leu Arg
95 100 105

Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys His Leu Pro
110 115 120

Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly Val Leu
125 130 135

Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg Leu
140 145 150

Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
155 160 165

Gly Cys Gly Val Thr Asp Lys Thr His Thr Cys Pro Pro Cys Pro
170 175 180

Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro

185 190 195

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
200 205 210

Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
215 220 225

Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
230 235 240

Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
245 250 255

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
260 265 270

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
275 280 285

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
290 295 300

Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
305 310 315

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
320 325 330

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
335 340 345

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
350 355 360

Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
365 370 375

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
380 385 390

Leu Ser Leu Ser Pro Gly Lys
395 397

CLAIMS:

1. A long half-life derivative of an OB protein retaining a biological property of a native OB protein.

2. The long half-life derivative of claim 1 capable of reducing body weight and/or food intake in an individual treated.

3. The derivative of claim 1 which is a derivative of a native human OB protein.

4. The derivative of claim 1 which is an OB-immunoglobulin chimera.

5. The derivative of claim 1 which is a native OB protein or an OB-immunoglobulin chimera modified with a nonproteinaceous polymer.

6. The derivative of claim 4 wherein the nonproteinaceous polymer is polyethylene glycol (PEG).

7. A composition for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising an effective amount of an OB derivative of claim 1.

8. The composition of claim 7 effective for weight and/or appetite reduction.

9. The composition of claim 7 effective in the reduction of elevated insulin levels.

10. A method for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising administering to an individual to be treated a derivative of claim 1.

11. The method of claim 10 wherein the condition to be treated is selected from the group consisting of obesity, bulemia, and Type I or II diabetes.

12. A method for inducing weight loss or appetite loss is a subject, comprising administering to said subject an effective amount of a derivative of claim 1.

13. A chimeric polypeptide comprising an OB protein amino acid sequence capable of binding to a native OB receptor, linked to an immunoglobulin sequence.

14. The chimeric polypeptide of claim 13 wherein said immunoglobulin sequence is a constant domain sequence.

15. The chimeric polypeptide of claim 14 wherein said OB protein is human.

16. The chimeric polypeptide of claim 15 wherein two OB polypeptide-IgG heavy chain fusions are linked to each other by at least one disulfide bond to yield a homodimeric immunoglobulin-like structure.

17. The chimeric polypeptide of claim 16 wherein at least one of said OB polypeptide-IgG heavy chain fusions is associated with an immunoglobulin light chain.

18. An isolated nucleic acid sequence encoding an OB protein-immunoglobulin fusion.

19. A replicable expression vector comprising the nucleic acid of claim 18.

20. A host cell transformed wih the replicable expression vector of claim 19.

21. A process comprising culturing the host cells of claim 16 so as to express the nucleic acid encoding an OB protein-immunoglobulin fusion.

22. The process of claim 21 wherein said host cells are cotransformed with nucleic acid encoding at least two OB protein-immunoglobulin fusions.

23. The process of claim 22 wherein said cells are further transformed with nucleic acid encoding at least one immunoglobulin light chain.

24. A method of treating a condition associated with the abnormal expression or function of the OB gene or for eliciting a biological response mediated by an OB receptor comprising administering to a patient a therapeutically effective amount of the chimeric polypeptide of claim 13.

25. The method of claim 20 wherein said condition is selected from the group consisting of obesity, bulemia and type I or II diabetes.

26. A composition for the treatment of obesity comprising an effective amount of a chimeric polypeptide of claim 13 in association with a pharmaceutically acceptable carrier.

27. A method for inducing the growth of cells expressing an OB receptor comprising contacting said cells with the OB derivative of claim 1.

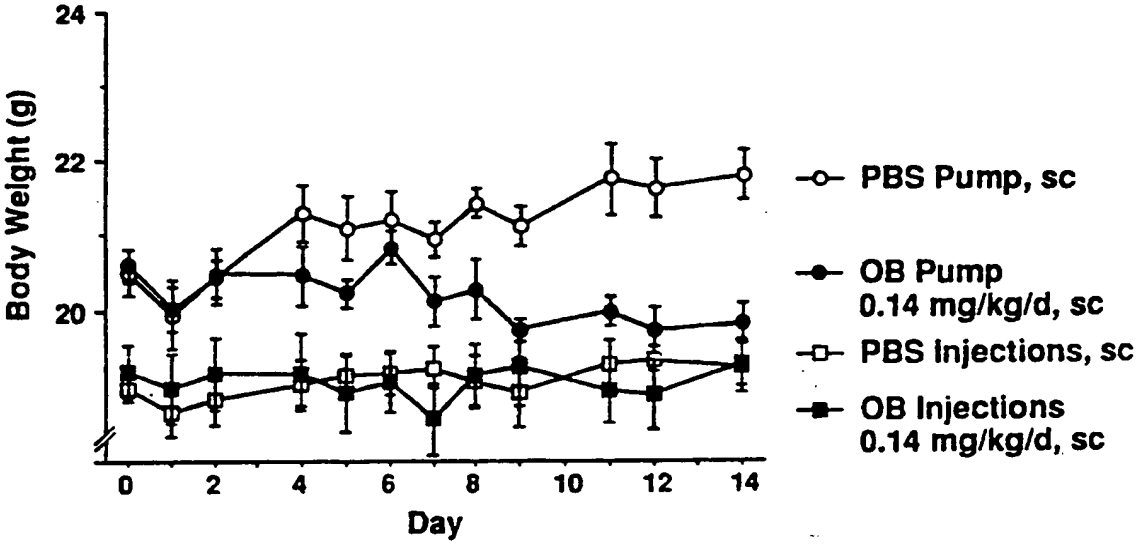

Body Weight (g)
24
22
20
0
2
4
6
8
10
12
14
Day
PBS Pump, sc
OB Pump
0.14 mg/kg/d, sc
PBS Injections, sc
OB Injections
0.14 mg/kg/d, sc

FIG. 1A

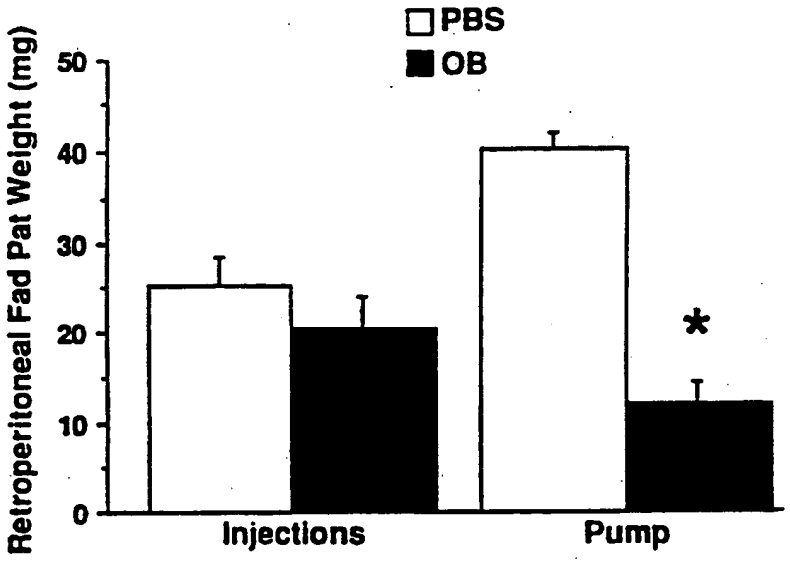

PBS
OB
Retroperitoneal Fad Pat Weight (mg)
50
40
30
20
10
0
*
Injections
Pump

FIG. 1B

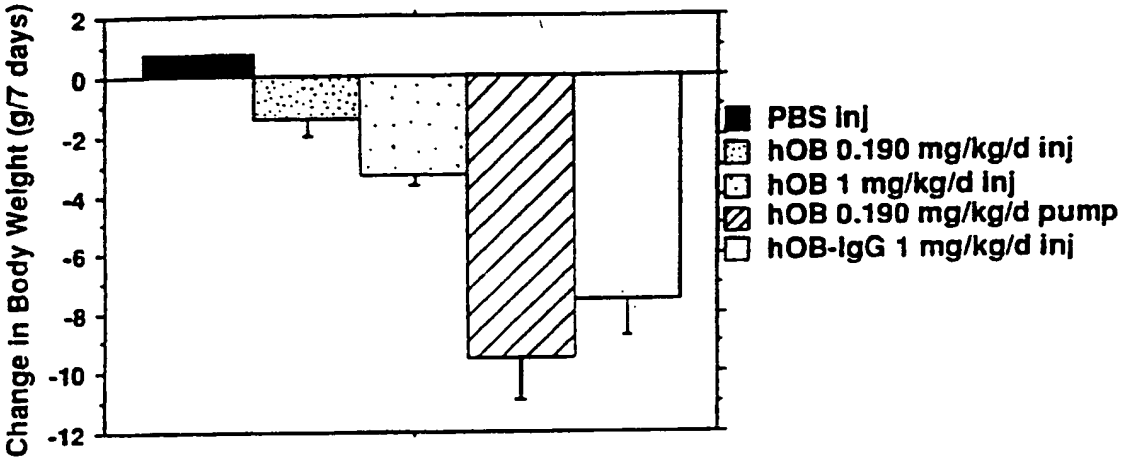

Change in Body Weight (g/7 days)
2
0
-2
-4
-6
-8
-10
-12
PBS inj
hOB 0.190 mg/kg/d inj
hOB 1 mg/kg/d inj
hOB 0.190 mg/kg/d pump
hOB-IgG 1 mg/kg/d inj

FIG. 2A

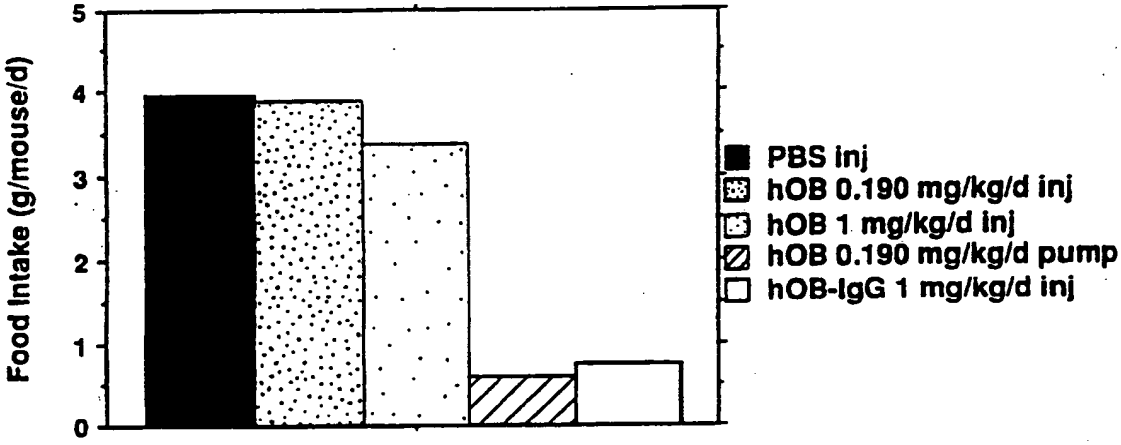

Food Intake (g/mouse/d)
5
4
3
2
1
0
PBS inj
hOB 0.190 mg/kg/d inj
hOB 1 mg/kg/d inj
hOB 0.190 mg/kg/d pump
hOB-IgG 1 mg/kg/d inj

FIG. 2B

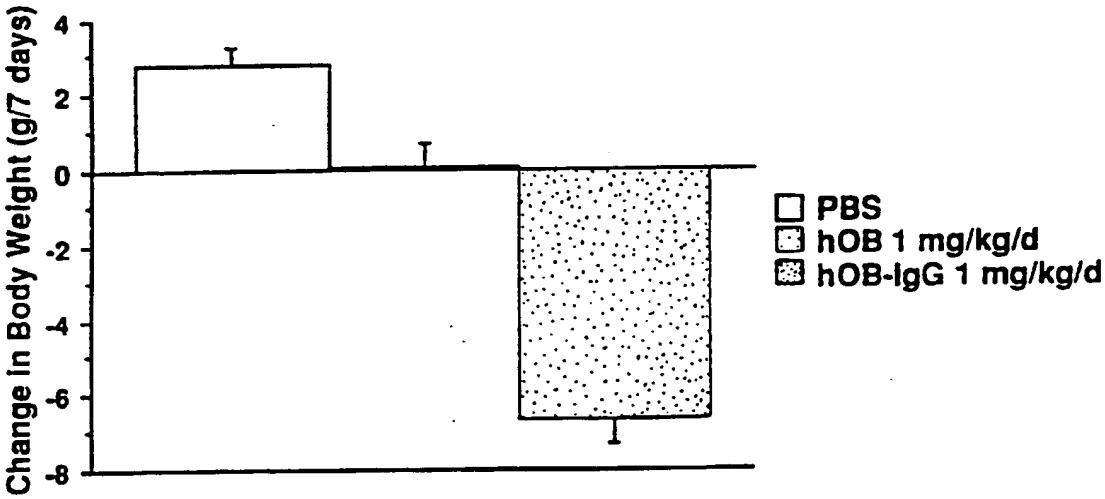

Change In Body Weight (g/7 days)
4
2
0
-2
-4
-6
-8
PBS
hOB 1 mg/kg/d
hOB-IgG 1 mg/kg/d

FIG. 3A

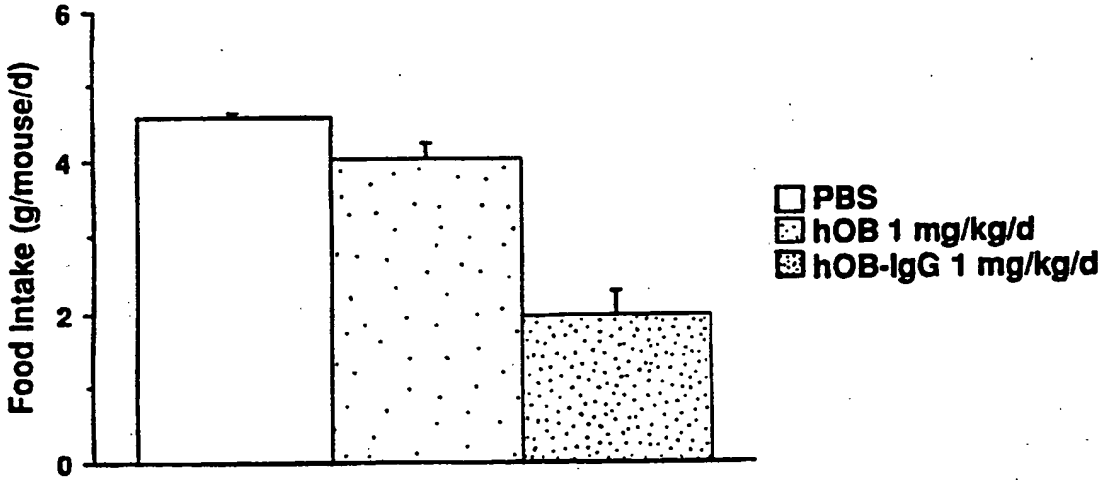

Food Intake (g/mouse/d)
6
4
2
0
PBS
hOB 1 mg/kg/d
hOB-IgG 1 mg/kg/d

FIG. 3B

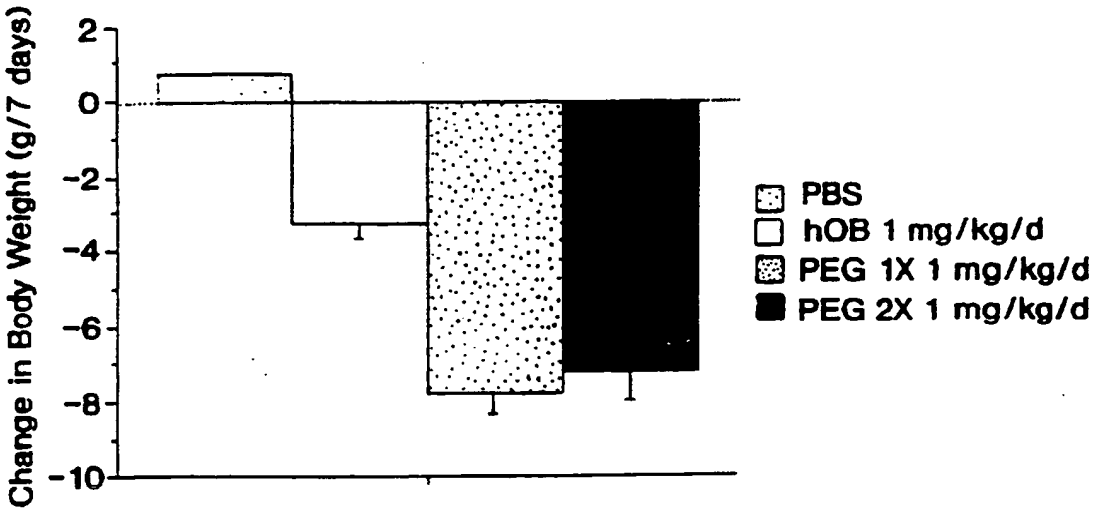

Change in Body Weight (g/7 days)
2
0
-2
-4
-6
-8
-10
PBS
hOB 1 mg/kg/d
PEG 1X 1 mg/kg/d
PEG 2X 1 mg/kg/d

FIG. 4A

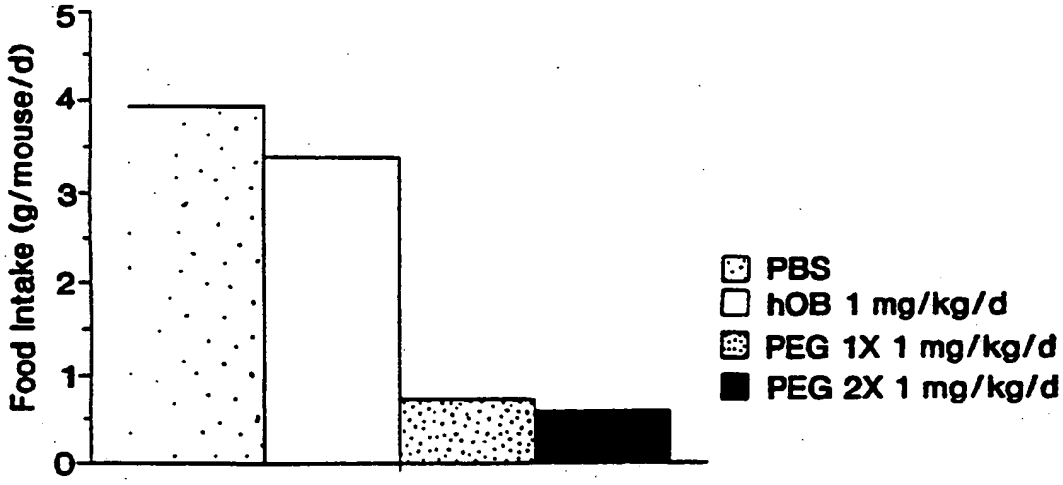

Food Intake (g/mouse/d)
5
4
3
2
1
0
PBS
hOB 1 mg/kg/d
PEG 1X 1 mg/kg/d
PEG 2X 1 mg/kg/d

FIG. 4B

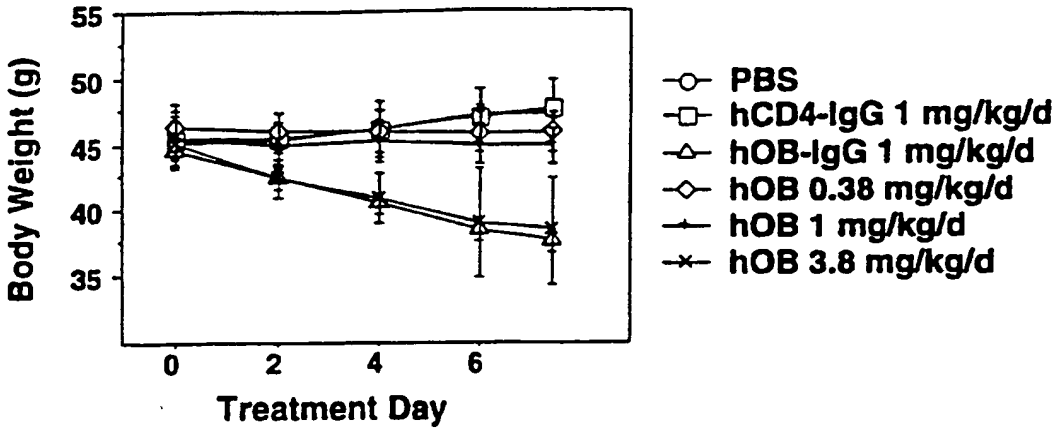


FIG. 5A

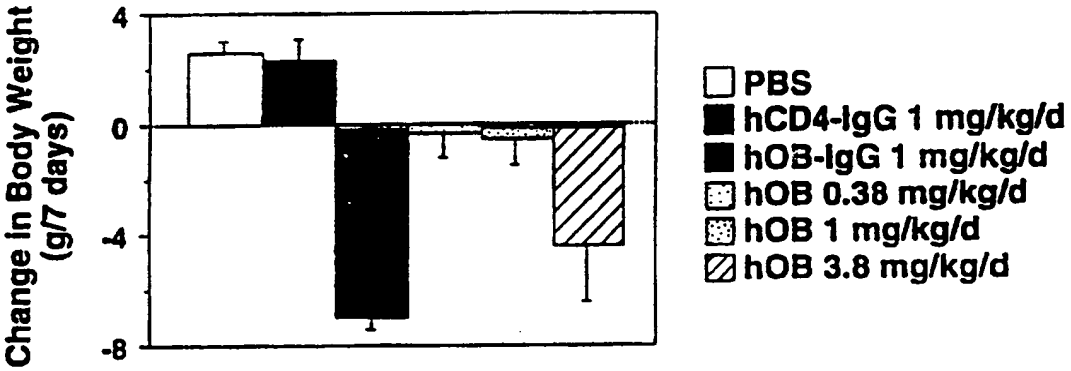


FIG. 5B

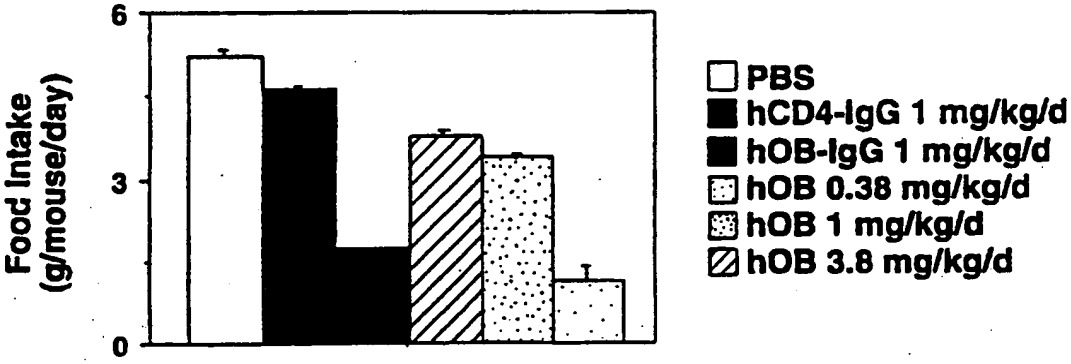


FIG. 5C

```
>                                ss.pRK5tkneo.hOBIgC
> sites: std
> length: 7127 (circular)
>
>human OB ClaI/BstEII cloning
>CMV enhancer/promoter

        aluI
       sstI
       sacI
       hgiJII
       hgiAI/aspHI
       ecl136II
       bsp1286                                                                                     thaI
       bsiHKAI                                                                                     fnuDII/mvnI
       bmyI                       rmaI    tru9I                                                    bstUI
       banII                      maeI    mseI                                                     bsh1236I
     taqI                        speI    aseI/asnI/vspI                           bslI            aciI maeIII
  1 TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC
    AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCAATAATTA TCATTAGTTA ATGCCCCAGT AATCAAGTAT CGGGTATATA CCTCAAGGCG CAATGTATTG

                      scrFI
                      mvaI
                      ecoRII
                      dsaV
                   aciI
                 bglI bstNI                                maeII
                sau96I                                   hinlI/acyI
                haeIII/palI     aciI                     ahaII/bsaHI
                asuI  apyI[dcm+]               aciI     aatII          maeII           maeIII
101 TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TCACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA
    AATGCCATTT ACCGGGCGGA CCGACTGGCG GGTTGCTGGG GGCGGGTAAC TGCAGTTATT ACTGCATACA AGGGTATCAT TGCGGTTATC CCTGAAAGGT

       maeII                                                                                      maeII
      hinlI/acyI                                                                                 hinlI/acyI
      ahaII/bsaHI                                     rsaI                    rsaI           ahaII/bsaHI
      aatII                         bglI         csp6I            ndeI       csp6I          aatII
201 TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT
    AACTGCAGTT ACCCACCTCA TAAATGCCAT TTGACGGGTG AACCGTCATG TAGTTCACAT AGTATACGGT TCATGCGGGG GATAACTGCA GTTACTGCCA
```

FIG. 6A

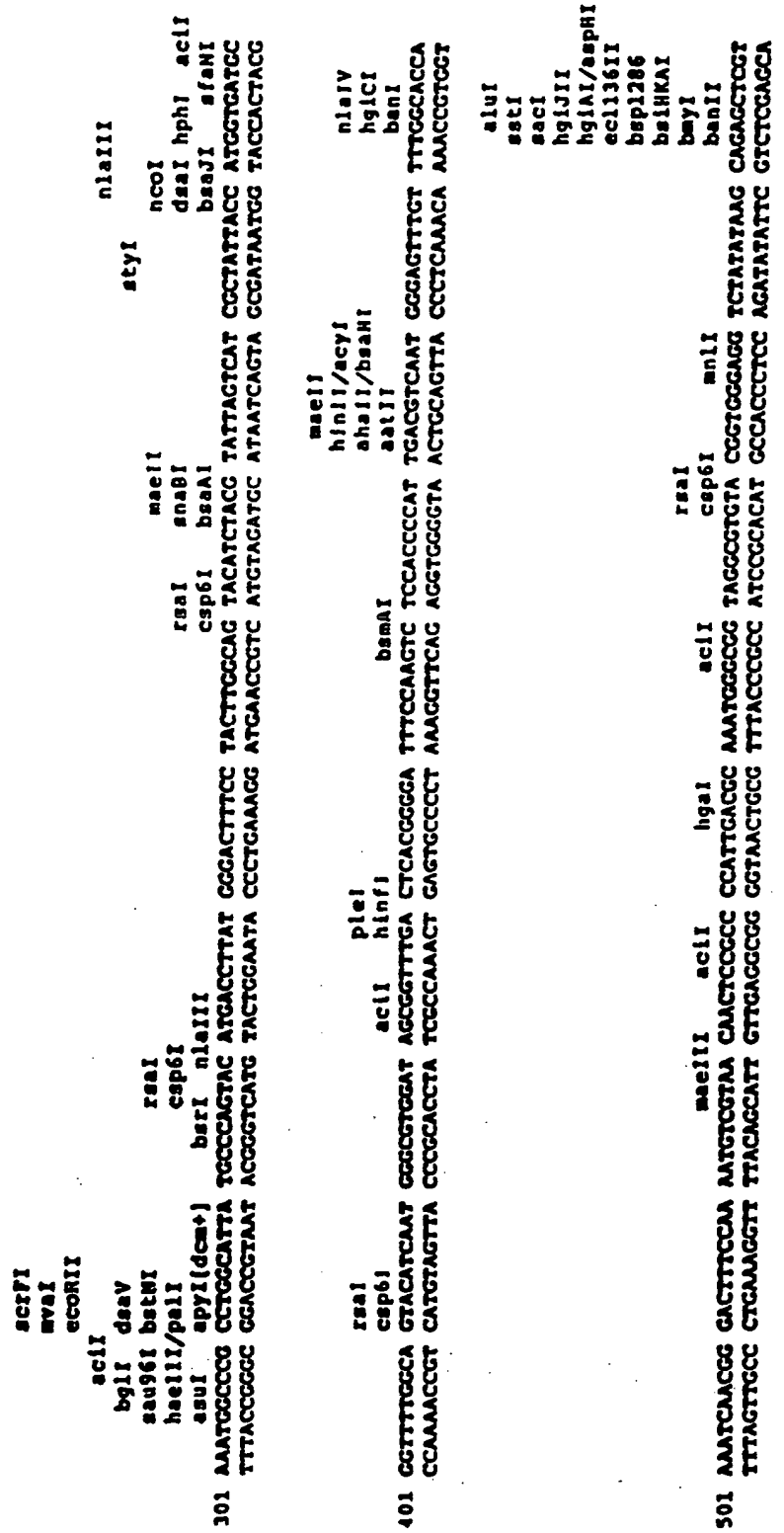

acrFI
mvaI
ecoRII
aclI
bglI dsaV
sau96I bstNI
hae111/pal1
asuI spyI(dcm+)
rsaI
csp6I
barI nla111
nla111
styI
ncoI
dsaI hphI aclI
bsaJI sfaNI
mae11
snaBI
bsaAI
rsaI
csp6I
301 AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT CGGACTTTCC TACTTGGCAG TATTAGTCAT CGCTATTACC ATGGTGATGC
TTTACCGGGC GGACCGTAAT ACGGGTCATG TACTGGAATA CCCTGAAAGG ATGAACCGTC ATGTAGATGC ATAATCAGTA GCGATAATGG TACCACTACG
mae11
hin11/acyI
aha11/bsaHI
at11
nlaIV
hgiCI
banI
rsaI
csp6I
aclI hinfI
pleI
bsmAI
401 GGTTTTGGCA GTACATCAAT GGGCGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA
CCAAAACCGT CATGTAGTTA CCCGCACCTA TCGCCAAACT GAGTGCCCCT AAAGGTTCAG AGGTGGGGTA ACTGCAGTTA CCCTCAAACA AAACCGTCGT
alu1
sstI
sacI
hgiJII
hgiAI/aspHI
acl1361I
bsp1286
bslHKAI
bayI
banII
rsaI
csp6I
mnlI
maeIII aclI hgaI aclI
501 AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT
TTTAGTTGCC CTGAAAGGTT TTACAGCATT GTTGAGGCGG GGTAACTGCG TTTACCCGCC ATCCGCACAT GCCACCCTCC AGATATATTC GTCTCGAGCA

FIG. 6B

```
                                                                                                haeIII/palI
                                                                                               mcrI
                                                                                               eagI/xmaIII/eclXI
                                                                                               eaeI
                                                                                               cfrI
                                                                                              fnu4HI
                                                                                              aciI
                                                                                             thaI
                             esp3I                                                           fnuDII/mvnI
                        scrFI                                            sau96I           sacII/sstII
                        mvaI bsmAI                                             avaII            nspBII
                        ecoRII                                           asuI             kspI  scrFI
                        dsaV                                            nlaIV             dsaI  ncil
                        bstNI  hinlI/acyI                                   scrFI          bglI bslI  mspI
                        apyI[dcm+]                                          ncil     sau3AI mnlI bstUI
                   sau3AI gsuI/bpmI                                         mspI     mboI/ndeII[dam-] hpaII
                   mboI/ndeII[dam-]                                 mboII   hpaII    dpnI[dam+] bsaJI dsaV
                   dpnI[dam+]  hgaI  fokI                           bpuAI   dsaV     dpnII[dam-] bsh1236I
                   dpnII[dam-] ahaII/bsaHI                 mnlI     bbsI    cauII    alwI[dam-] aciI  cauII
601 TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA
    AATCACTTGG CAGTCTAGCG GACCTCTGCG GTAGGTGCGA CAAAACTGGA GGTATCTTCT GTGGCCCTGG CTAGGTCGGA GGCGCCGGCC CTTGCCACGT
                 ^Begin RNA

               tfiI                                                                             fnu4HI
           aciI                                                                                 aciI
          thaI hinfI                                                  bstXI                    thaI
          fnuDII/mvnI                         aciI                sau96I    styI               fnuDII/mvnI tru9I
          bstUI                          maeII rsaI           pleI scfI haeIII/palI            bstUI           mseI
          bsh1236I                   maeIII    csp6I   scfI hinfI     asuI      bsaJI          bsh1236I   aseI/asnI/vspI
701 TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA
    AACCTTGCGC CTAAGGGGCA CGGTTCTCAC TGCATTCATG GCGGATATCT CAGATATCCG GGTGGGGGAA CCGAAGCAAT CTTGCGCCGA TGTTAATTAT
                                                                                      ^sp6 promoter
```

FIG. 6C

sau96I
AvaII
AsuI
ScrFI
NvaI
EcoRII
DsaV
BstNI
BslI BsaJI
801 CATAACTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
GTATTGAAT ACATAGTATG TGTATGCTAA ATCCACTGTG ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG
^sp6 RNA start
MaeIII
HphI ScfI FokI
PpuMI TfiI Sau96I
MnlI TaqI NsiI/AvaIII HinfI HaeIII/PalI Bsp1286
BsaJI ClaI/Bsp106 NlaIV AciI AsuI AluI BmyI FokI
901 ACCTCGGTTC TATCGATATG CATTGGGGAA CCCTGTGCGG ATTCTTGTGG CTTTGGCCCT ATCTTTTCTA TGTCCAAGCT GTGCCCATCC AAAAGTCCA
TGGAGCCAAG ATAGCTATAC GTAACCCCTT GGGACACGCC TAAGAACACC GAAACCGGGA TAGAAAAGAT ACAGGTTCGA CACGGGTAGG TTTTTCAGGT
1 Met HisTrpGlyT hrLeuCysGl yPheLeuTrp LeuTrpProT yrLeuPheTy rValGlnAla ValProIleG lnLysValGln
^cloning linker ^human OB start
Sau3AI
MboI/NdeII[dam-]
DpnI[dam+]
ScrFI
NvaI
EcoRII
DsaV
BstNI
SpyI[dcm+]
HphI DpnI[dam-]
MnlI
MunI MaeIII AlwI[dam-]
1001 AGATGACACC AAAACCCTCA TCAGACAAAT TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC AGAAAGTCAC CGGTTTGGAC
TCTACTGTGG TTTTGGGAGT AGTTCTGTTA ACAGTGGTCC TAGTTACTGT AAAGTGTGTG CGTCAGTCAG AGGAGGTTTG TCTTTCAGTG GCCAAACCTG
29 AspAspThr LysThrLeuI leLysThrIl eValThrArg IleAsnAspI leSerHisTh rGlnSerVal SerSerLysG lnLysValTh rGlyLeuAsp
MspI
HpaII
CfrI0I
BsaWI
AgeI
HphI
MaeIII
BsmAI
MnlI

FIG. 6D

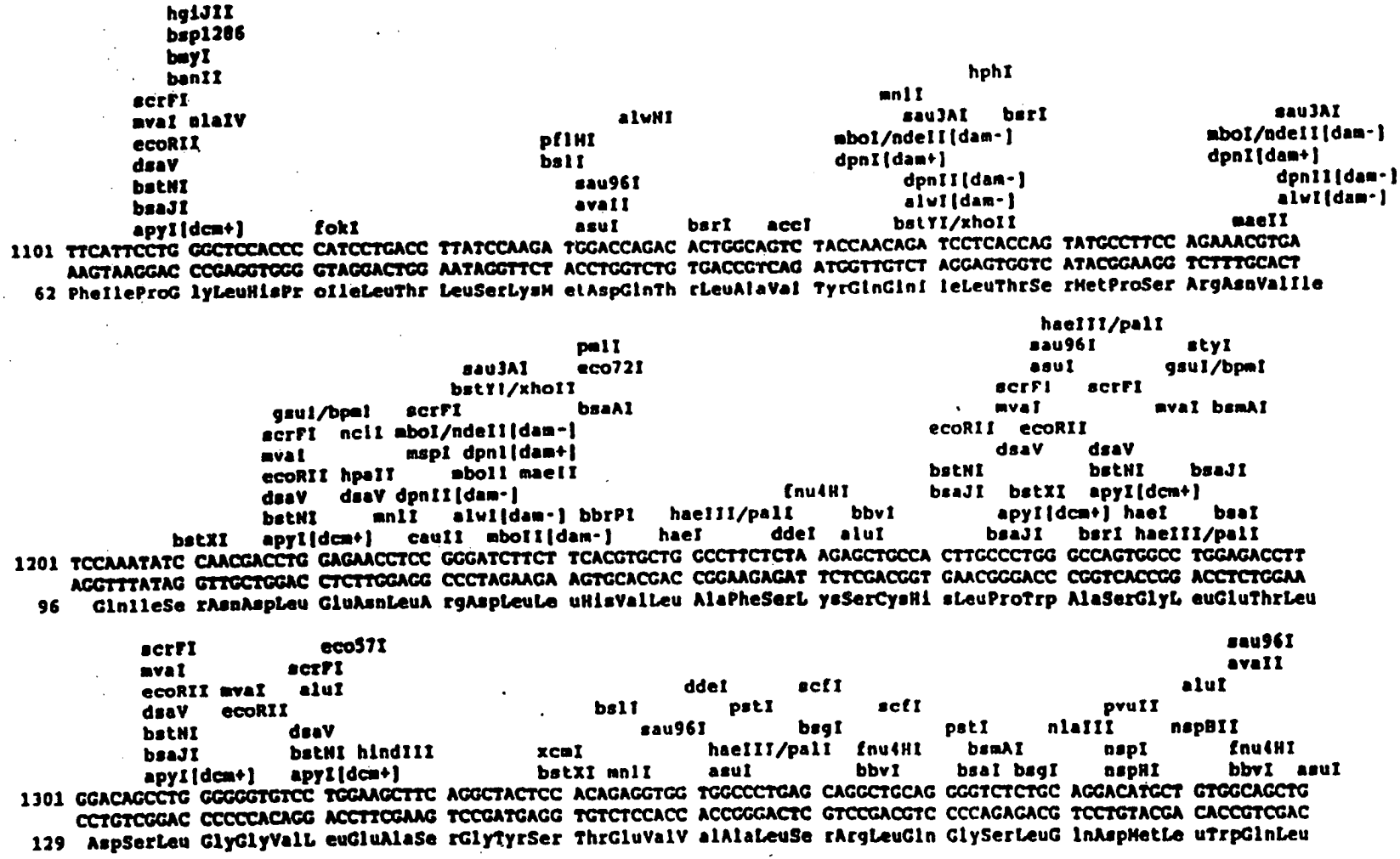

hgiJII
bsp1286
bmyI
bsnII
scrFI
mvaI sfaIV
ecoRII
dsaV
bstNI
bsaJI
apyI[dcm+]
fokI
alwNI
pflMI
bslI
sau96I
avaII
asuI
bsrI
accI
hphI
mnlI
sau3AI
bsrI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
alwI[dam-]
bstYI/xhoII
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
alwI[dam-]
maeII
1101 TTCATTCCTG GGCTCCACCC CATCCTGACC TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG TATGCCTTCC AGAAACGTGA
AAGTAAGGAC CCGAGGTGGG GTAGGACTGG AATAGGTTCT ACCTGGTCTG TGACCGTCAG ATGGTTGTCT AGGAGTGGTC ATACGGAAGG TCTTTGCACT
62 PheIleProG lyLeuHisPr oIleLeuThr LeuSerLysM etAspGlnTh rLeuAlaVal TyrGlnGln leLeuThrSe rMetProSer ArgAsnValIle
pmlI
sau3AI
eco72I
bstYI/xhoII
gsuI/bpmI
scrFI
bsaAI
haeIII/palI
sau96I
styI
asuI
gsuI/bpmI
scrFI
scrFI
mvaI
mvaI bsmAI
scrFI nciI mboI/ndeII[dam-]
mvaI mspI dpnI[dam+]
ecoRII hpaII mboII maeII
dsaV dsaV dpnII[dam-]
bstNI mnlI alwI[dam-] bbrPI
fnu4HI
haeIII/palI
bbvI
ecoRII ecoRII
dsaV dsaV
bstNI bstNI bsaJI
bsaJI bstXI apyI[dcm+]
apyI[dcm+] haeI bsaI
bstXI apyI[dcm+] cauII mboII[dam-] haeI ddeI aluI
bsaJI bsrI haeIII/palI
1201 TCCAAATATC CAACGACCTG GAGAACCTCC GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG GCCAGTGGCC TGGAGACCTT
AGGTTTATAG GTTGCTGGAC CTCTTGGAGG CCCTAGAAGA AGTGCACGAC CGGAAGAGAT TCTCGACGGT GAACGGGACC CGGTCACCGG ACCTCTGGAA
96 GlnIleSe rAsnAspLeu GluAsnLeuA rgAspLeuLe uHisValLeu AlaPheSerL ysSerCysHi sLeuProTrp AlaSerGlyL euGluThrLeu
scrFI
mvaI
ecoRII mvaI
dsaV ecoRII
bstNI
bsaJI
apyI[dcm+]
eco57I
scrFI
aluI
dsaV
bstNI hindIII
xcmI
bstXI mnlI
ddeI
bslI
sau96I
haeIII/palI
asuI
scfI
pstI
bsgI
fnu4HI
bbvI
scfI
pstI
bsmAI
bsaI
pvuII
nlaIII
bsgI
nspI
nspHI
sau96I
avaII
aluI
nspBII
fnu4HI
bbvI asuI
1301 GGACAGCCTG GGGGGTGTCC TGGAAGCTTC AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC AGGACATGCT GTGGCAGCTG
CCTGTCGGAC CCCCCACAGG ACCTTCGAAG TCCGATGAGG TGTCTCCACC ACCGGGACTC GTCCGACGTC CCCAGAGACG TCCTGTACGA CACCGTCGAC
129 AspSerLeu GlyGlyValL euGluAlaSe rGlyTyrSer ThrGluValV alAlaLeuSe rArgLeuGln GlySerLeuG lnAspMetLe uTrpGlnLeu

FIG. 6E

eam1105I
sau96I
scrFI
mvaI
ecoRII
dsaV
bstNI
bslI
bsaJI
hphI
nlaIII
nspI
bsp1286
nspHI
bmyI alwNI
baeI nlaIV
apyI(dcm+)
bstEII
aclI
maeIII
ddeI
mnlI
GACCTCAGCC CTGGGTGCGG GGTCACCGAC AAACTCACA CATGCCCACC GTGCCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC CTCTTCCCCC
CTGGAGTCGG GACCCACGCC CCAGTGGCTG TTTTGAGTGT GTACGGGTGG CACGGGTCGT GGACTTGAGG ACCCCCTGG CAGTCAGAAG GAGAAGGGGG
AspLeuSerP roGlyCysGl yValThrAsp LysThrHisT hrCysProPr oCysProAla ProGluLeuL euGlyGlyPr oSerValPhe LeuPheProPro
^insertion of a gly
^START OF HUMAN IgG1 CH2CH3
sau96I
nlaIV
nspI
hpaII
scrFI
nciI
dsaV
sau3AI
mboI/ndeII(dam-)
nlaIII
rcaI
bspHI(dam-)
styI
bsaJI
dpnII(dam-)
ecoRI
mnlI
bsu36I/mstII/sauI
caeIII
nspI
nspHI
maeIII
ddeI
caeII
mnlI
mboII
bpuAI
eco81I
bbsI
drdI
ddeI
bsu36I/mstII/sauI
maeII
rsaI
cap6I
barI
bsaAI
1401
1501
162
196
GTCAGTCTTC CTCTTCCCCC
CAGTCAGAAG GAGAAGGGGG
bpuAI earI/ksp632I
bbsI mnlI
mboII
ACGTGAGCCA CGAAGACCCT GAGGTCAAGT TCAACTGGTA
TGCACTCGGT GCTTCTGGGA CTCCAGTTCA AGTTGACCAT
sPvalSerHi sGluAspPro GluValLysP heAsnTrpTyr
GTCCCAGCA CCTGAACTCC TGGGGGGACC
CACGGGTCGT GGACTTGAGG ACCCCCTGG
ProGluLeuL euGlyGlyPr oSerValPhe
bmyI alwNI
baeI nlaIV
apyI(dcm+)
GTGCCCAGCA CCTGAACTCC TGGGGGGACC
CACGGGTCGT GGACTTGAGG ACCCCCTGG
ProGluLeuL euGlyGlyPr oSerValPhe

FIG. 6F

acil
thaI
fnuDII/mvnI
bstUI
bsh1236I
sacII/sstII
nspBII
kspI
dsaI
bsaJI
acil
rsaI
csp6I
bsaAI
maeII
hgaI
hphI
mnlI
ecoNI
bstNI
scrFI
mvaI
ecoRII
dsaV
bslI
apyI(dcm+)
fnu4HI
mnlI
csp6I
fnu4III
bbvI
avaI
1601 CGTGGACGGC GTGGAGGTGC ATAATGCCAA GACAAAGCCG CGGGACGAGC AGTACAACAG CACGTACCGT GTGGTCAGCG TCCTCACCGT CCTGCACCAG
GCACCTGCCG CACCTCCACG TATTACGGTT CTGTTTCGGC GCCCTCCTCG TCATGTTGTC GTGCATGGCA CACCAGTCGC AGGAGTGGCA GGACGTGGTC
229 ValAspGly ValGluValH isAsnAlaLy sThrLysPro ArgGluGluG lnTyrAsnSe rThrTyrArg ValValSerV alLeuThrVa lLeuHisGln
rsaI
bsaAI
mnlI
csp6I
bsaI
taqI
bbvI
avaI
1701 GACTGGCTGA ATGGCAAGGA GTACAAGTGC AAGGTCTCCA ACAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCATCTCCAA AGCCAAAGGG CAGCCCCGAG
CTGACCGACT TACCGTTCCT CATGTTCACG TTCCAGAGGT TGTTTCGGGA GGGTCGGGGG TAGCTCTTTT GGTAGAGGTT TCGGTTTCCC GTCGGGGCTC
262 AspTrpLeuA snGlyLysG luTyrLysCys LysValSerA snLysAlaLe uProAlaPro IleGluLyst hrIleSerLy sAlaLysGly GlnProArgGlu
scrFI
ncil
mspI
hpaII
dsaV
cauII
xmaI/papAI
smaI
scrFI
ncil
dsaV
cauII
fokI
bslI bsaJI mboII
bslI aval earI/ksp632I
scrFI
nvaI
ecoRII
dsaV
bstNI
apyI(dcm+)
sexAI
scrFI
mvaI
ecoRII
dsaV
bstNI
apyI(dcm+)
bspHI
rsaI
csp6I
bsp1407I
1801 AACCACAGGT GTACACCCTG CCCCCATCCC GGGAAGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCCC
TTGGTGTCCA CATGTGGGAC GGGGGTAGGG CCCTTCTCTA CTGGTTCTTG GTCCAGTCGG ACTGGACGGA CCAGTTTCCG AAGATAGGGT CGCTGTAGCC
296 ProGlnVa lTyrThrLeu ProProSerA rgGluGluMe tThrLysAsn GlnValSerL euThrCysLe uValLysGly PheTyrProS erAspIleAla

FIG. 6G

```
                                  mspI
                                  hpaII                                                                    dsaI
                               fnu4HI                                    pleI                  mnlI          bphI
                               bbvI                          mnlI        hinfI     nlaIV  mboII   scfI     aluI  bsaJI
1901 CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTACAG CAAGCTCACC
     GCACCTCACC CTCTCGTTAC CCGTCGGCCT CTTGTTGATG TTCTGGTGCG GAGGGCACGA CCTGAGGCTG CCGAGGAAGA AGGAGATGTC GTTCGAGTGG
 329  ValGluTrp GluSerAsnG lyGlnProGl uAsnAsnTyr LysThrThrP roProValLe uAspSerAsp GlySerPheP heLeuTyrSe rLysLeuThr

                                                                                                       scrFI
                                                                                                       ncil
                                     mboII                                                             mspI
                                     bpuAI                  nlaIII                                     hpaII
                                   maeII                  ppu10I                          sapI        dsaV
                          fnu4HI   xmnI bbsI               nsiI/avaIII                     mboII mnlI      bsmAI
                   bspMI  bbvI     asp700      nlaIII     sfaNI   mnlI                    earI/ksp632I bslI   cauII
2001 GTGGACAAGA GCAGGTGGCA GCAGGGGAAC GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC TCCCTGTCTC
     CACCTGTTCT CGTCCACCGT CGTCCCCTTG CAGAAGAGTA CGAGGCACTA CGTACTCCGA GACGTGTTGG TGATGTGCGT CTTCTCGGAG AGGGACAGAG
 362 ValAspLysS erArgTrpGl nGlnGlyAsn ValPheSerC ysSerValHe tHisGluAla LeuHisAsnH isTyrThrGl nLysSerLeu SerLeuSerPro

                                                                    sau96I
                                                                   nlaIII
                                     taqI                        fnu4HI   haeIII/palI
                                     salI                  taqI  bglI styI
                                   pleI     scfI           pleI     scfI  sfiI  ncoI
                                 rmaI hincII/hindII       rmaI  salI  pstI  eaeI   dsaI
                          sau96I  hinfI    pstI          xbaI   hincII/hindII    cfrI   bsaJI                  aluI
                          haeIII/palI      bsgI    aluI maeI  acci  bsgI    aluI haeIII/palI                fnu4HI
                          asuI maeI acci bspMI   hindIII   hinfI  bspMI    hindIII  aciI   asuI                bbvI
2101 CGGGTAAATG AGTGCGACGG CCCTAGAGTC GACCTGCAGA AGCTTCTAGA GTCGACCTGC AGAAGCTTGG CCGCCATGGC CCAACTTGTT TATTGCAGCT
     GCCCATTTAC TCACGCTGCC GGGATCTCAG CTGGACGTCT TCGAAGATCT CAGCTGGACG TCTTCGAACC GGCGGTACCG GGTTGAACAA ATAACGTCGA
 396   GlyLys
                                                                                                  ^sv40 early poly A

                                                                  rmaI
          maeIII              sfaNI   apoI                      bsmI maeI
2201 TATAATGGTT ACAAATAAAG CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC ATCAATGTAT
     ATATTACCAA TGTTTATTTC GTTATCGTAG TGTTTAAAGT GTTTATTTCG TAAAAAAAGT GACGTAAGAT CAACACCAAA CAGGTTTGAG TAGTTACATA
```

FIG. 6H

sau3AI
mboI/ndeII(dam-)
dpnI(dam+)
dpnII(dam-)
pvuI/bspCI
mcrI
taqI(dam-) tru9I
haeIII/palI
haeI
claI/bsp106(dam-)
fnu4HI styI
sau3AI nseI
bbvI ncoI
mboI/ndeII(dam-)
hinPI dsaI
dpnI(dam+) xmnI
dpnII(dam-) asel/asnI/vspI bsaJI
bsp718 snlI
rsaI
csp6I
nlaIV
kpnI
hgiCI
banI
acc65I ddeI aclI
snlI
mnlI
nlaIII alwI(dam-) asp700 hhaI/cfoI nlaIII
origin
^sv40
nlaIII
CTTATCATGT CTGGATCCAT CGGCAATTAA TTCGGCCCAG CACCATGGCC TGAAATAACC TCTGAAAGAG GAACTTGGTT AGGTACCTTC TGAGGCGGAA
GAATAGTACA GACCTAGGTA GCCGTTAATT AAGCCGGGTC GTGGTACCGG ACTTTATTGG AGACTTTCTC CTTGAACCAA TCCATGGAAG ACTCCGCCTT
nlaIV
scrFI
mvaI
ecoRII
dsaV
bstNI
apyI(dcm+)
bsaJI
sfaNI
ppuI0I
nsiI/avaIII
nlaIII
sphI
nspI
nspHI
scrFI
mvaI
ecoRII
dsaV
bstNI
apyI(dcm+)
sexAI
aluI
pvuII
nspBII
GTCGAATGTG TGTCAGTTAG GTGTGCAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC
TCTTGGTCGA CACTTACAC ACAGTCAATC CCACACTTT CAGGGGTCCG AGGGGTCGTC CGTCTTCATA CGTTTCGTAC GTAGAGTTAA TCAGTCGTTG
nlaIV
scrFI
mvaI
ecoRII
dsaV
bstNI
apyI(dcm+)
bsaJI
ppuI0I
nsiI/avaIII
nlaIII
sphI
nspI sfaNI
nspHI
aclI
aclI fokI
GAGGTGTCGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC
GTCCACACCT TTCAGGGGTC CGAGGGGTCG TCCGTCTTCA TACGTTTCGT ACGTAGAGTT AATCAGTCGT TGGTATCAGG GCGGGGATTG AGGCGGGTAG

FIG. 6I

fnu4HI
bglI
afiI
haeIII/palI
mnlI mnlI ddeI
haeIII/palI bsaJI mnlI aluI
mnlI bsaJI aclI haeIII/palI
nlaIII
styI
ncoI
bslI dsaI
bsrI acsI acII bsaJI
acII
2601 CGGCCCCTAA CTCCGCCCAG TTCCCCCCAT TCTCCGCCCC ATGCCTGACT AATTTTTTTT ATTTATGCAG AGGCCCAGGC CGCCTCGGCC TCTGAGCTAT
GGCCGGGATT GAGGCGGGTC AAGGCGGGGG TACCGACTGA TTAAAAAAAA TAAATACGTC TCCGGCTCCG GCGGACCCGG AGACTCGATA
scrFI
mvaI
sau96I
nlaIV
avaII sau96I
thaI ecoRII
fnuDII/mvnI
bstUI dsaV
bsh1236I avaII
hinPI bstNI
hhaI/cfoI asuI
fnu4HI asuI apyI[dcm+]
acII acII bsaJI
styI sfuI sfaNI
bsaJI bstBI
binI bsiCI
haeIII/palI asuII
stuI rmaI tru9I
haeI maeI
mnlI alwI taqI
mnlI mnlI aluI maeI taqI
2701 TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTGTTAATTC GAACACGCAG ATGCAGTCGG GGCGCCCCGG TCCCAGGTCC
AGGTCTTCAT CACTCCTCCG AAAAACCTC CGGATCCGAA AACGTTTTTC GACAATTAAG CTTGTGCGTC TACGTCAGCC CCGCGCGCCC AGGGTCCAGG
^start pUC118
^TK promoter
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
bstYI/xhoII
bglII dpnII[dam-]
fnu4HI bclI[dam-]
thaI
fnuDII/mvnI
bstUI
mluI fnu4HI
afIIII bbvI
hphI bsh1236I mnlI scfI
tru9I hgaI haeIII/palI pstI tru9I hincII/hindII acII dpnII[dam-] bsmAI
msel maeIII haeI taqI bsgI acII msel hgaI fnu4HI
2801 ACTTCGCATA TTAAGGTGAC GCGTGTGGCC TCGAACACCG AGCGACCCTG CAGCGACCCG CTTAACAGCG TCAACAGCGT GCCGCAGATC TGATCAAGAG
TGAAGCGTAT AATTCCACTG CGCACACCGG AGCTTGTGGC TCGCTGGGAC GTCGCTGGGC GAATTGTCGC AGTTGTCGCA CGGCGTCTAG ACTAGTTCTC
tn5 neomycin phosphotransferase gene. ^

FIG. 6J

acli
fnu4HI
haeIII/pali
ncrI
eagI/xmaIII/eclXI
eaeI
cfrI
mspI
bsp1286
bmyI
bspHI
hpaII
mnlI
barI
hinPI
hhaI/cfoI
nlaIV
bsp1286
narI scrFI
bmyI
kasI nciI
nlaIV
hinlI/acyI
hgiCI
hgiCI mspI
banI
haeII hpaII
banI dsaV
mspI
acfI
hpaII
patI
hinPI ahaII/bsaHI
drdI
bsaWI
bagI
acli
fnu4HI
mspI
hhaI/cfoI caulI
hpaII
bbvI
sfaNI
bali
fnu4HI
bsp1286
alui
pvuII
fnu4HI
bbvI bsiHKAI
hinPI bmyI
hhaI/cfoI
maeII
eco57I
mstI nspBII
tthlIII/aspI
avaII/fspI
taqI
maeIII
aciI
barI bbvI
fnu4HI
thaI
fnuDII/mvnI
bstUI
bsh1236I
hinPI
fnu4HI
bbvI aciI
mnlI hhaI/cfoI
haeIII/paII
mscI/baII
haeI
eaeI
cfrI
3001 AGACAATCGG CTGCTCTGAT GCCCCCGTGT TCCCGCTGTC AGCGCAGGGG CGCCCGGTTC TTTTTGTCAA GACCGACCTG TCCCGTGCCC TGAATGAACT
TCTGTTAGCC GACGAGACTA CGGCGGCACA AGGGCGACAG TCGCGTCCCC GCGGGCCAAG AAAAACAGTT CTGGCTGGAC AGGGCACGGG ACTTACTTGA
3101 GCAGGACGAG GCAGCGCGGC TATCGTGGCT GGCCACGACG GGCGTTCCTT GCGCAGCTGT GCTCGAGGTT GTCACTGAAG CGGGAAGGGA CTGGCTGCTA
CGTCCTGCTC CGTCGCGCCG ATAGCACCGA CCGGTGCTGC CCGCAAGGAA CGCGTCGACA CGACGTCCAA CAGTGACTTC GCCCTTCCCT GACCGACGAT

FIG. 6K

3201 TTGGGCGAAG TGCCGGGGCA GCATCTCCTG TCATCTCACC TTGCTCCTGC CGAGAAAGTA TCCATCATGG CTGATGCAAT GCGGCGCCTG CATACGCTTG
AACCCGCTTC ACGGCCCCGT CGTAGAGGAC AGTAGAGTGG AACGAGGACG GCTCTTTCAT AGGTAGTACC GACTACGTTA CGCCGCCGAC GTATGCGAAC
3301 ATCCGGCTAC CTGCCCATTC GACCACCAAG CGAAACATCG CATCGAGCGA GCACGTACTC GGATGGAAGC CGGTCTTGTC GATCAGGATG ATCTGGACGA
TAGGCCGATG GACGGGTAAG CTGGTGGTTC GCTTTGTAGC GTAGCTCGCT CGTGCATGAG CCTACCTTCG GCCAGAACAG CTAGTCCTAC TAGACCTGCT
3401 AGAGCATCAG GGGCTCGCGC CAGCCGAACT GTTCGCCAGC CTCAAGCCGC GCATGCCCGA CGGCAGGAT CTCGTCGTGA CCCATGGCGA TGCCTGCTTG
TCTCGTAGTC CCCGAGCGCG GTCGGCTTGA CAAGCGGTCC CGTACGGGCT GCCGCTCCTA GAGCAGCACT GGGTACCGCT ACGGACGAAC

FIG. 6L

```
                                                       mspI             bslI
                                                       hpaII           sau96I
             aciI                                     naeI             avaII
            fnu4HI                                    cfr10I           asuI
           haeIII/palI                               haeIII/palI      rsrII/cspI
          eaeI           tfiI                       eaeI              cpoI
   nlaIII cfrI           hinfI    taqI              cfrI              aciI aciI
3501 CCGAATATCA TGGTGGAAAA TGGCCGCTTT TCTGGATTCA TCGACTGTGG CCGGCTGGGT GTGGCGGACC GCTATCAGGA CATAGCGTTG GCTACCCGTG
     GGCTTATAGT ACCACCTTTT ACCGGCGAAA AGACCTAAGT AGCTGACACC GGCCGACCCA CACCGCCTGG CGATAGTCCT GTATCGCAAC CGATGGGCAC

   sapI                                                                            hinPI
   mboII       fnu4HI                                          bsrBI               hhaI/cfoI
   earI/ksp632I                                                aciI     tfiI  fnu4HI
 eco57I aluI   aciI          acrI     mnlI                  fnu4HI     hinfI bbvI sfaNI
3601 ATATTGCTGA AGAGCTTGGC GGCGAATGGG CTGACCGCTT CCTCGTGCTT TACGGTATCG CCGCTCCCGA TTCGCAGCGC ATCGCCTTCT ATCGCCTTCT
     TATAACGACT TCTCGAACCG CCGCTTACCC GACTGGCGAA GGAGCACGAA ATGCCATAGC GGCGAGGGCT AAGCGTCGCG TAGCGGAAGA TAGCGGAAGA

                           taqI
                          sfuI
           aciI           bstBI               hinlI/acyI                  tfiI      aciI
       ddeI      pleI     bsiCI               hgaI     bspMI              hinfI    fnu4HI
   mboII  bsrBI  hinfI    asuII               ahaII/bsaHI              taqI     aciI
3701 TGACGAGTTC TTCTGAGCGG GACTCTGGGG TTCGAAATGA CCGACCAAGC GACGCCCAAC CTGCCATCAC GAGATTTCGA TTCCACCGCC GCCTTCTATG
     ACTGCTCAAG AAGACTCGCC CTGAGACCCC AAGCTTTACT GGCTGGTTCG CTGCGGGTTG GACGGTAGTG CTCTAAAGCT AAGGTGGCGG CGGAAGATAC
```

FIG. 6M

scrFI
ncil
mspI
hpaII
dsaV
cauII
bsll
xmaI/pspAI
smaI
scrFI
nciI
dsaV
cauII
bseJI
bseJI
HSV1 tk terminator SmaI-PvuII
mboII bsll avaI
GCCCACCCCG GGACATGGGG
CGGGTGGGGC CCTCTACCCC
TGCCCACAAC CCAGCAAACA
AAAAGACAG AATAAAACGC ACGGGTGTTG GGTCGTTTGT
TTTTTCTGTC TTATTTTGCG
thaI
hae111/pall fnuDII/mvnI
sau96I bstUI
asuI bsh1236I
nlaIV acil mboII
thaI
hinPI
hhaI/cfoI
thaI
fnuDII/mvnI
bstUI
bsh1236I
nlaIV acil
ACCCCGCGTA TACGCCCGCG TTTCTTCCTT TTCCCCACCC
ATGCGCGCGC AAAGAAGGAA AAGGGGTGGG
hinPI
hhaI/cfoI
thaI
fnuDII/mvnI
bstUI
bsh1236I
nlaIV acil
TGACGCAATT AAAAGACAG AATAAAACGC ACGGGTGTTG GGTCGTTTGT
ACTGCCCTTA TTTTTCTGTC TTATTTTGCG TGCCCACAAC CCAGCAAACA
scrFI
mvaI
ecoRII
dsaV
bstMI
bseJI
bsll
sau96I
nlaIV
acil
thaI
fnuDII/mvnI
bseJI
bsmAI
asuI
nlaIV
bsaI
GATACCCCAC CGAGACCCCA TTCGCCCCGC TTTCTTCCTT TTCCCCACCC
CTATGGGGTG GCTCTGGGGT AACCCCGGTT ATGCGCGCGC AAAGAAGGAA AAGGGGTGGG
bsmAI nlaIV acil
GGGGTTCGGT CCCAGGGCTG GCACTCTGTC GATACCCCAC CGAGACCCCA TTCGCCCCGC TTTCTTCCTT TTCCCCACCC
CCCCAAGCCA CGGTCCCGAC CGTCAGACAG CTATGGGGTG GCTCTGGGGT AACCCCGGTT ATGCGCGCGC AAAGAAGGAA AAGGGGTGGG

FIG. 6N

hgiJII
bsp1286
bmyI
scrFI
nvaI
ecoRII
dsaV
bstNI
bsaJI
apyI(dcm+)
bsaJI
sau96I bsII
bsII hae3II/paII fnu4HI
bsII hphI asuI banII bbvI
4101 CAACCCCAA GTTCGGGTGA AGGCCCAGGG CTCGCAGCCA ACGTCGGGGC GGCAAGCCCG CCATAGCCAC GGGCCCCGTG GGTTAGGGAC GGGGTCCCCC
GTTGGGGGTT CAAGCCCACT TCCGGGTCCC GAGCGTCGGT TCCAGCCCCG CCGTTCCGGC GGTATCGGTG CCCGGGGCAC CCAATCCCTG CCCCAGGGGG
sau96I bsII
hae3II/paII fnu4HI
asuI banII bbvI hae3I
4201 ATGGGAATG GTTTATGGTT CGTGGGGGTT ATTCTTTTGG GCGTTGCGTG GGGTCAGGTC CACGACTGGA CTGAGCAGAC AGACCCATGG TTTTTGGATG
TACCCTTAC CAATACCAA GCACCCCAA TAAGAAAACC CGCAACGCAC CCCAGTCCAG CTGCTGACCT GACTCGTCTG TCTGGGTACC AAAAACCTAC
hinII/acyI ahaII/basHI
scrFI sau96I scrFI
nvaI thaI ncII
ecoRII rsaI fnuDII/mvnI
dsaV ava3I nla3III batUI aspI
bstNI asuI aspI hinPI hpaII
bsII bsaJI nla3III aspHI barI bah1236I dsaV hgaI fnu4HI
apyI(dcm+) acII csp6I hha1/cfoI cauII bbvI
4301 GCCTGGGCAT GGACCGCATG TACTGGCGCG ACAGAACAC CGGGCGTCTG TGGCTGCCAA ACACCCCCGA CCCCCAAAAA CCACCCCCCG GATTTCTGGC
CGGACCCGTA CCTGGCGTAC ATGACCGCGC TGTCTTGTG GCCCCCAGAC ACCGACGGTT TGTGGGGGCT GGGGGTTTTT GGTGGGGGGC CTAAAGACCG
thaI
fnuDII/mvnI hinPI
batUI hha1/cfoI
bah1236I nla3V narI
hinPI
thaI kasI
fnuDII/mvnI hin1I/acyI
batUI hgiCI
bah1236I haeII
acII banI
bsII hha1/cfoI aha3II/basHI

FIG. 6O

aclI
thaI
fnuDII/mvnI
batUI
sacII/satII
haeIII/palI bsh1236I
mcrI
nspBII
dsaI
kspI
bsaJI
dsaI
hphI eagI/xmaIII/eclXI
maeIII eaeI
bsaJI
bstZII cfrI
aclI
hinPI
mnlI hhaI/cfoI
rsaI haeII
csp6I eco47III
GGTACCAGGA GCGCTTTTGT TTTGTATTGG TCACCACGGC CGAGTTTCCG
CCATGGTCCT CGCGAAAACA AAACATAACC AGTGGTGCCG GCTCAAAGGC
bslI sfaNI mboII
GAACTAAACC TGACTACGGC ATCTCTGCCC CTTCTTCGCT
CTTGATTTGG ACTGATGCCG TAGAGACGGG GAAGAAGCGA
scrFI nlaIV
ncII hgiCI
dsaV scrFI
cauII sva1
bslI ecoRII
bslI dsaV
bsaJI bstNI
sau96I bsaJI
nlaIV haeIII/palI
sva1I eaeI
asuI cfrI bsp1286
ppuMI mspI apyI[dcm+]
nlaIV hpaII bmyI
eco01091/draII banI
GCCAGGGCAC CTGTCCTACG AGTTGCATGA TAAAGATGAC AGTCATAAGT
CGGTCCCGTG GACAGGATGC TCAACGTACT ATTTCTTCTG TCAGTATTCA
hinPI mspI
hhaI/cfoI
thaI hpaII
fnuDII/mvnI
bstUI baeNI
bsh1236I
fnu4HI
aclI bcgI nlaIII aciI bsiI aluI
GCGGCGACGA TAGTCATGCC CCGCGCCCAC CGGAAGGAGC
CGCCGCTCCT ATCAGTACGG GGCGCGGGTG GCCTTCCTCG
^pBR322 sequence

FIG. 6P

fnu4HI
haeIII/paII
ncrI
eagI/xmaIII/eclXI
eaeI
notI
fnu4HI
aclI
ncrI bsrBI aclI
sfaNI taqI cfrI sfaNI
bsrI
4601 TGACTGGGTT GAAGCTCTC AAGGCATCG GTCGAGCGGC CGCATCAAAG CAACCATAGT ACGCGCCCTG TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT
ACTGACCCAA CTTCCGAGAG TTCCGTAGC CAGCTCGCCG GCGTAGTTTC GTTGGTATCA TGCGCGGGAC ATCGCCGCGT AATTCGCGCC GCCCACACCA
^delta 3
^M13 ori
fnu4HI
hinPI
hhaI/cfoI
thaI
fnuDII/mvnI
bstUI
bsh1236I aclI
maeIII bbvI maeIII
4701 GGTTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT
CCAATGCGCG TCGCACTGCC GATGTGAACG GTCGCGGGCG CGAAGCGAAA GAAGCGAAGG AAAGAGCGGT GCAAGCGGCC GAAAGGGGCA
nlaIV
hgiJII
bsp1286
bmyI
banII
alwI
4801 CAAGCTCTAA ATCGGGGGCT CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC CCCAAAAAAC TTGATTTGGG TGATGGTTCA CGTAGTGGGC
GTTCGAGATT TAGCCCCCGA GGGAAATCCC AAGCTAAATT CACGAAATGC CGTGGAGCTG GGGTTTTTTG AACTAAACCC ACTACCAAGT GCATCACCCG
maeII pleI
drdI hinfI maeII
bsrI
4901 CATCGCCCTG ATAGACGGTT TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT CCAAACTGGA ACAACACTCA ACCCTATCTC
GTAGCGGGAC TATCTGCCAA AAAGCGGGAA ACTGCAACCT CAGGTGCAAG AAATTATCAC CTGAGAACAA GCTTTGACCT TGTTGTGAGT TGGGATAGAG
tru9I pleI
hinfI
bsrI
bslI
bslI aveI
haeIII/paII
sau96I
asuI
ncoI
hphI
draIII
bseAI
maeII
FIG. 6Q

```
                                                                                            thaI
                                                                                            fnuDII/mvnI
                                                    tru9I                           tru9I   apoI tru9I
                                                    mseI              tru9I         mseI bstUI  mseI
                                            haeIII/palI         aluI  mseI    apoI  bsh1236I         sspI
5001 GGGCTATTCT TTTGATTTAT AAGGGATTTT GCCGATTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA CAAAAATTTA ACGCGAATTT TAACAAAATA
     CCCGATAAGA AAACTAAATA TTCCCTAAAA CGGCTAAAGC CGGATAACCA ATTTTTTACT CGACTAAATT GTTTTTAAAT TGCGCTTAAA ATTGTTTTAT

       maeII                 mnlI                                                           maeII
      psp1406I             haeIII/palI                        nlaIII                        hinlI/acyI
     tru9I                 stuI                      tru9I rcaI                             ahaII/bsaHI
     mseI                  haeI                      mseI  bspHI                      ddeI  aatII
5101 TTAACGTTTA CAATTTTATG GTGCAGGCCT CGTGATACGC CTATTTTTAT AGGTTAATGT CATGATAATA ATGGTTTCTT AGACGTCAGG TGGCACTTTT
     AATTGCAAAT GTTAAAATAC CACGTCCGGA GCACTATGCG GATAAAAATA TCCAATTACA GTACTATTAT TACCAAAGAA TCTGCAGTCC ACCGTGAAAA
                             ^delta 2a

                nlaIV
               aciI
              thaI
              fnuDII/mvnI
              bstUI
              bsh1236I                                                rcaI
             hinPI                                                    bspHI
             hhaI/cfoI                                            bsrBI   bsmAI
                                                                  aciI nlaIII                           sspI
5201 CGGGGAAATG TGCGCGGAAC CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG CTTCAATAAT
     GCCCCTTTAC ACGCGCCTTG GGGATAAACA AATAAAAAGA TTTATGTAAG TTTATACATA GGCGAGTACT CTGTTATTGG GACTATTTAC GAAGTTATTA

            mboII                                               fnu4HI
            earI/ksp632I                                        aciI                          hphI
5301 ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT
     TAACTTTTTC CTTCTCATAC TCATAAGTTG TAAAGGCACA GCGGGAATAA GGGAAAAAAC GCCGTAAAAC GGAAGGACAA AAACGAGTGG GTCTTTGCGA

                                  hgiAI/aspHI
                                  bsp1286                         sau3AI
                      sau3AI      bsiHKAI                         mboI/ndeII[dam-]   sau3AI
                      mboI/ndeII[dam-]                            dpnI[dam+]         mboI/ndeII[dam-]
                      dpnI[dam+] bmyI                             dpnII[dam-]        dpnI[dam+]
                      dpnII[dam-]                                bstYI/xhoII         dpnII[dam-]
                 eco57I           apaLI/snoI                  bsrI        nspBII     alwI[dam-]
     hphI      sfaNI mboII[dam-]  alw44I/snoI  maeIII taqI  alwI[dam-] aciI    bstYI/xhoII          mboII
5401 GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA
     CCACTTTCAT TTTCTACGAC TTCTAGTCAA CCCACGTGCT CACCCAATGT AGCTTGACCT AGAGTTGTCG CCATTCTAGG AACTCTCAAA AGCGGGGCTT
```

FIG. 6R

scrFI
nclI
mspI
hpaII
dsaV
caulI
aclI
thaI
fnuDII/mvnI
bstUI
bsh1236I
hinPI
hhaI/cfoI
hinlI/acyI
hgaI
ahaII/bsaHI
aclI
bcgI
mcrI fnu4HI
hg1AI/aspHI
bsp1286 tru9I
bsiHKAI msei
bmyI ahaIII/draI
fnu4HI
bbvI
nlaIII
rsaI
csp6I barI
scaI hphI maeIII
sfaNI
fokI nlaIII
nlaIII
sau3AI maeIII
mboI/ndeII[dam-]
dpnI[dam+]
alwI[dam-]
dpnII[dam-]
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
sau96I
avaII
sau3AI asuI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
pvuI/bspCI
mcrI mnlI
alwI aclI
nlaIII dpnII[dam-]
hinPI
mstI
avIII/fspI barI
maeII hhaI/cfoI tru9I
psp1406I mseI
maeIII sfaNI
fnu4HI
bbvI
haeIII/paII
eaeI
cfrI
fnu4HI
aclI
mspI
hpaII
bsaHI
nlaIV aluI
5501 GAACGTTTTC CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT GATCACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT
CTTGCAAAAG GTTACTACTC GTGAAAATTT CAAGACGATA CACCGCGCCA TAATAGGGCA CTACTGCGGC CCGTTCTCGT TGAGCCAGCG GCGTATGTGA
5601 ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAGCATCTTA CGGATGGCCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG
TAAGAGTCTT ACTGAACCAA CTCATGAGTG GTCAGTGTCT TTTCGTAGAA TGCCTACCGT ACTGTCATTC TCTTAATACG TCACGACGGT ATTGGTACTC
5701 TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT
ACTATTGTGA CGCCGGTTGA ATGAAGACTG TTGCTAGCCT CCTGGCTTCC TCGATTGGCG AAAAACGTTG TTGTACCCCC TAGTACATTG AGCGGAACTA
5801 CGTTGGGAAC CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCA GCAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG
GCAACCCTTG GCCTCGACTT ACTTCGGTAT GGTTTGCTGC TCGCACTGTG GTGCTACGGT CGTCGTTACC GTTGTTGCAA CGCGTTTGAT AATTGACCGC

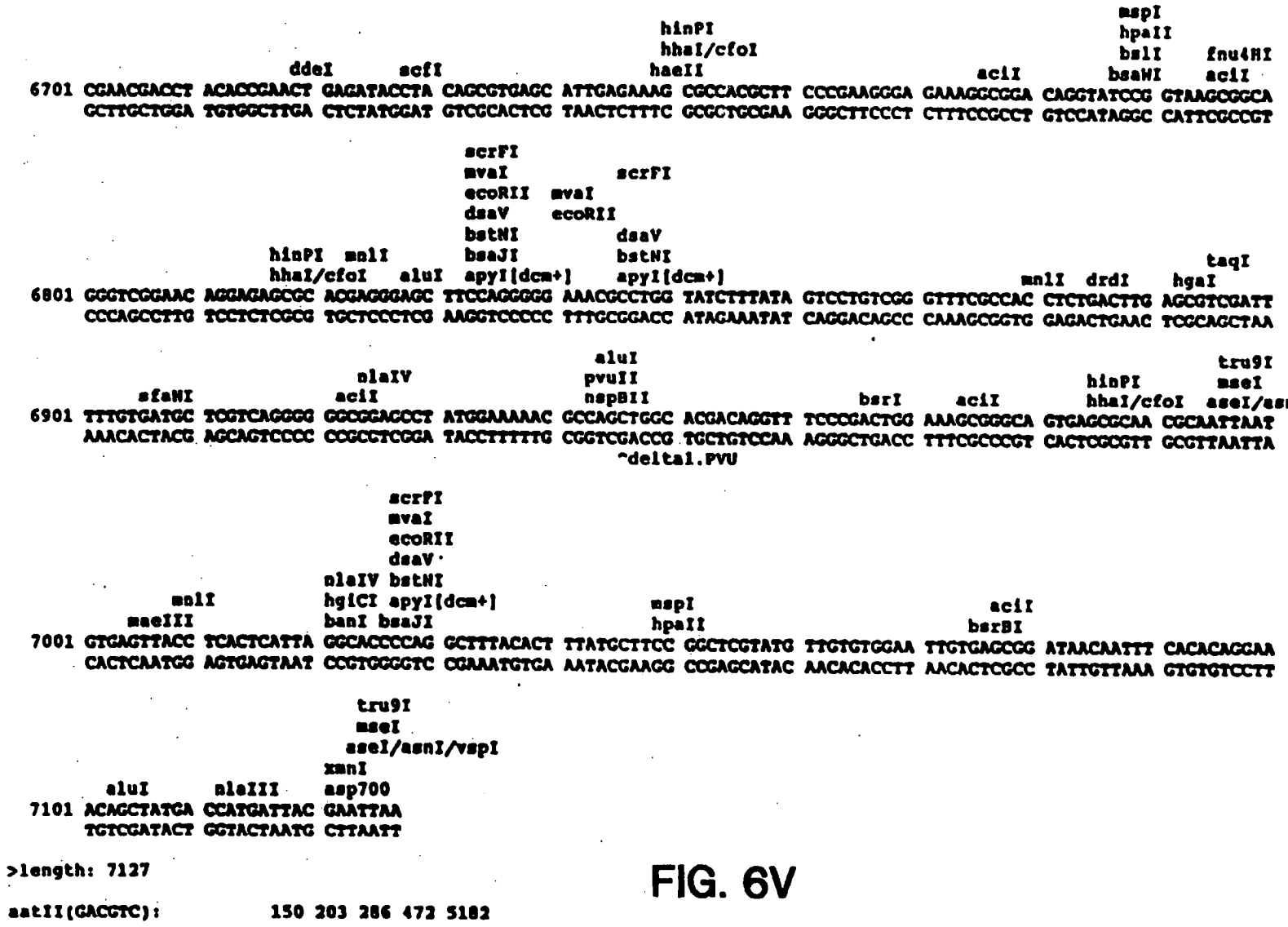
FIG. 6S

bglI
sau96I
haeIII/paII
hinPI asuI mspI
hhaI/cfoI hpaII
sau96I
avaII
asuI
mnlI
eam1105I
GTATCGTAGT TATCTACACG
CATAGCATCA ATAGATGTGC
mspI
hpaII
cfr10I
nlaIV hphI
gsuI/bpmI
fnuDII/mvnI
bstUI
bsaI bsh1236I
sau96I
asuI
fnu4HI nlaIV
bbvI bsrI haeIII/paII
AAGCCCTCCC
TTCGGGAGGG
ddeI
sau3AI nlaIV
mboI/ndeII[dam-] mnlI
dpnI[dam+] hgiCI tru9I
dpnII[dam-] banI maeI
maeIII
GTCAGACCAA GTTTACTCAT
CAGTCTGGTT CAAATGAGTA
pleI
hinfI
fokI
ACGGGGAGTC AGCCAACTAT GGATGAACGA AATAGACAGA TCGCTCAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT
TGCCCCTCAG TCCGTTGATA CCTACTTGCT TTATCTGTCT AGCGACTCTA TCCACGGAGT GACTAATTCC TAACCATTGA
hphI
rmaI sau3AI
sau3AI mboI/ndeII[dam-]
mboI/ndeII[dam-]
dpnI[dam+] dpnI[dam+]
dpnII[dam-] dpnII[dam-]
tru9I
ahaIII/draI maeI
alwI[dam-]
nlaIII
maeII
tru9I
maeI
ahaIII/draI
tru9I bstYI/xhoII
maeI maeI alwI[dam-] mboII[dam-]
bstYI/xhoII
rcaI
bspHI
tru9I
maeI
ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT
TATATGAAAT CTAACTAAAT TTTGAAGTAA AAATTAAATT TTCCTAGATC CACTTCTAGG AAAAACTATT AGAGTACTGG TTTTAGGGAA TTGCACTCAA

FIG. 6T

sau3AI
mboI/ndeII(dam-)
dpnI(dam+) sau3AI thaI
dpnII(dam-) mboI/ndeII(dam-)
bstYI/xhoII dpnI(dam+) fnuDII/mvnI
sau3AI alwI(dam-) dpnII(dam-) bstUI
mboI/ndeII(dam-) alwI(dam-) bsh1236I
dpnI(dam+) mboII(dam-) hinPI fnu4HI
ddeI hgaI dpnII(dam-) bstYI/xhoI hhaI/cfoI bbvI
6301 TTCGTTCCAC TGAGCGTCAG ACCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA
AAGCAAGGTG ACTCGCAGTC TGGGCATCT TTTCTAGTTT CCTAGAAGAA CTCTAGCAAA AAAGACGCGG CATTAGACGA CGAACGTTTG TTTTTTTGGT
sau3AI
mboI/ndeII(dam-)
dpnI(dam+)
dpnII(dam-)
alwI(dam-)
aciI mspI
aciI nspBII hpaII aluI
6401 CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC
GGCGATGGTC GCCACCAAAC AAACGGCCTA GTTCTCGATG GTTGAGAAAA AGGCTTCCAT TGACCGAAGT CGTCTCGCGT CTATGGTTTA TGACAGGAAG
bsrI hinPI
maeIII eco57I hhaI/cfoI
fnu4HI
alwNI bbvI
bsrI fnu4HI
maeIII bbvI bsrI
haeIII/paII
scfI aciI smlI
ball haeI
6501 TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTCCTG CCAGTGGCGA
ATCACATCGG CATCAATCCG GTGGTGAAGT TCTTGAGACA TCGTGGCGGA TGTATGGAGC GACGATTA CGACAATGGT CACCGACGAC GGTCACCGCT
scrFI
ncil
nspI
hpaII
dsaV pleI
cauII hinfI
scrFI
nspBII
fnu4HI
bbvI
hinP1 aciI
haeIII hhaI/cfoI
hgiAI/aspHI
bsp1286
bslHKAI
bmyI
apaLI/snoI
alw44I/snoI aluI
6601 TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG
ATTCAGCACA GAATGGCCCA ACCTGAGTTC TGCTATCAAT GGCCTATTCC GCGTCGCCAG CCCGACTTGC CCCCCAAGCA CGTGTGTCGG GTCGAACCTC

FIG. 6U



>length: 7127

aatII(GACGTC): 150 203 286 472 5182

FIG. 6V

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C07K14/575 A61K38/22 C12N15/70 C12N1/21
//(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 00319 A (SMITHKLINE BEECHAM PLC ;BROWNE MICHAEL JOSEPH (GB); CHAPMAN CONRAD) 3 January 1997 see page 1, line 31 - line 33; claims; examples ---	1-4,7-26
P,X	EP 0 741 187 A (HOFFMANN LA ROCHE) 6 November 1996 see page 9, line 19 - page 11, line 46; claims; examples 19,20 ---	1-3, 6-12,26
P,X	WO 96 05309 A (UNIV ROCKEFELLER ;FRIEDMAN JEFFREY M (US); ZHANG YIYING (US); PROE) 22 February 1996 see page 43, line 3 - page 46, line 14; claims --- -/--	1-3, 6-12,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 1997	14.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20718

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 372, no. 6505, 1 December 1994, pages 425-432, XP000602062 YIYING ZHANG ET AL: "POSITIONAL CLONING OF THE MOUSE OBESE GENE AND ITS HUMAN HOMOLOGUE" see the whole document -----	1,10-12, 26

1

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 96/ 20718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7-10,24-25
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7-10, and 24-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20718

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9700319 A	03-01-97	AU 6011096 A	15-01-97
EP 0741187 A	06-11-96	AU 5197896 A	14-11-96
		CA 2175298 A	06-11-96
		ES 2093593 T	01-01-97
		JP 9003098 A	07-01-97
		NO 961796 A	06-11-96
		PL 314051 A	12-11-96
WO 9605309 A	22-02-96	AU 3329895 A	07-03-96
		CA 2195955 A	22-02-96
		DE 19531931 A	07-03-96
		FI 970656 A	17-02-97
		GB 2292382 A	21-02-96
		JP 9502729 T	18-03-97
		ZA 9506868 A	09-04-96